Parasite mediated selection, sex and diapause in a natural population of *Daphnia*

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Abstract

Parasites are thought to have large effects on their host populations, driving genetic change, population density changes, speciation and be a major selective force maintaining sexual reproduction. Indirect signatures of parasite-mediated selection are common, but explicit examples of parasite-mediated selection in nature are lacking. In this thesis I examine parasite-mediated dynamics in a natural population of *Daphnia magna* that experiences an annual epidemic of the bacterial pathogen *Pasteuria ramosa*. I also test a novel hypothesis investigating the relationship between parasitism and the production of resting eggs.

In chapter 2 a combined field study and laboratory infection experiment illustrates one of the best examples of parasite-mediated selection in a natural population, with *Daphnia* collected after a parasite epidemic having higher levels of parasite resistance than those collected before. This chapter also explored the relationship between parasitism and resting eggs, which are only produced during the sexual phase of reproduction. *Daphnia* that were reproducing sexually in the field prior to the parasite epidemic were more susceptible, supporting higher levels of parasite growth, than their asexual counterparts. This supports the idea that some genotypes invest in sex at the expense of parasite resistance.

In chapter 3 I used molecular markers to investigate genotype frequency changes in the same population in relation to the parasite epidemic. The parasite epidemic was found to be associated with genetic change in the population, and a laboratory infection experiment revealed that the genotype most resistant to the parasite was also most common following the peak of the parasite epidemic.

While chapter 2 explored a genetic relationship between susceptibility and resting eggs, chapter 4 explores whether crowding conditions, cues indicating parasite

prevalence in the population, or direct exposure to parasite spores can induce resting egg production. I found that crowding conditions or parasite prevalence enhanced levels of male and resting egg production, but patterns were entirely dependent on *Daphnia* genotypes. There was no indication that exposure to parasite spores affects levels of sexual reproduction.

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Declaration

I declare that this thesis has been composed by myself and is entirely my own work, except for the collaborative input mentioned below:

Chapter 2. Experiment 2 was carried out with the help of Sarah Proctor, a Zoology Honours student.

Chapter 4. The experiment was carried out with the help of Sharron Meaden, a Nuffield summer student.

Appendix 1. Experiment A1.2.1 and Experiment A1.2.2. were carried out with the help of Sarah Hall, a Zoology Honours student.

The following paper has arisen from work described in this thesis:

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Chapter 1. General Introduction

1.1. Parasite-mediated dynamics in natural populations

Parasites, by nature, are harmful to their hosts. Hosts, in response, have evolved ways to evade parasite-induced harm. Host-parasite relationships are thus predicted to be in a continuous flux of antagonistic co-evolution (Ebert & Herre, 1996; Haldane, 1949; Little, 2002; Woolhouse *et al.*, 2002). Parasites are therefore thought to be a major driving force for genetic change in their host populations, and may even select for recombination in hosts because this creates novel genotypes to which the parasite population is not yet adapted (Haldane, 1949; Hamilton *et al.*, 1990; Jaenike, 1978; Peters & Lively, 1999). However, studies of wild populations have frequently failed to demonstrate parasite-mediated genetic change (Henter, 1995; Little & Ebert, 2001; Mitchell *et al.*, 2004; Siemens & Roy, 2005), although indirect signatures of the impact of parasitism have commonly been identified in many natural systems. For example, genetic variation for infection related traits, such as resistance, a pre-requisite for parasite-mediated selection, is abundant in host populations (Henter, 1995; Koskela *et al.*, 2002; Kraaijeveld & Godfray, 1999; Little & Ebert, 2000b; Madsen & Ujvari, 2006). Furthermore patterns of genetic variation that are compatible with some forms of parasite-mediated dynamics (in particular parasite-mediated frequency dependent selection) have been identified in some populations (Dybdahl & Lively, 1995; Ebert, 1994; Lively & Dybdahl, 2000), but not others (Burdon & Thompson, 1995; Kaltz *et al.*, 1999; Little & Ebert, 2001; Siemens & Roy, 2005). Links between breeding system and parasite prevalence have also been identified (Busch *et al.*, 2004; Lively, 1987; Lively & Jokela, 2002), but see (Ben-

Ami & Heller, 2005; Meirmans *et al.*, 2006; Tobler & Schlupp, 2005) for cases where this has not been observed.

Clearer evidence for the occurrence of parasite-mediated selection comes from artificial, or contrived associations between hosts and parasites. For example, parasite-mediated selection has been demonstrated in experimental populations (Buckling & Rainey, 2002; Capaul & Ebert, 2003; Haag & Ebert, 2004), in populations exposed to artificial selection (Ibrahim & Barrett, 1991) and in biological control programmes, in which parasites have been used to control host populations that are considered to be pests (Shea *et al.*, 2000).

1.2. Why is parasite-mediated selection so elusive?

Detecting parasite-mediated selection in the field has been difficult for a number of reasons. Aside from environmental and genetic factors, there are also practical implications that must be considered. For example, the generation time of many taxa limits our ability to monitor selection through time, and in addition to this, the scale of any investigation is affected both by space constraints and the availability and costs of labour. Nevertheless, it is surprising the fact that there is little evidence of observed parasite-mediated dynamics in nature, especially considering the ubiquity of parasites and the frequently strong detrimental effects they have on their hosts. Moreover, some theoretical models do predict that the effects of parasitism must be severe for parasite-mediated frequency dependent selection to maintain sexual reproduction (Howard & Lively, 1994; May & Anderson, 1983; Otto & Nuismer, 2004).

The inability or failure of studies to consider a sufficient number of the factors that influence the relationship between hosts and parasites may account for the lack of evidence for parasite mediated selection. Indeed, host population dynamics are

affected by numerous environmental factors in addition to parasitism (Saccheri & Hanski, 2006 and references therein). Recent work has sought to gain insight into parasite-mediated dynamics by highlighting how environmental variation may interact with infection, and by examining how the genetic basis of infection may interact with the environment. Environmental variation has been shown to be a major factor determining the ability of a host, or its offspring, to defend against parasites (Little *et al.*, 2003; Mitchell *et al.*, 2005b; Moret & Schmid-Hempel, 2001; Robb & Forbes, 2005; Sadd *et al.*, 2005), and several studies have demonstrated strong genotype by environment (Fels & Kaltz, 2006; Ferguson & Read, 2002; Mitchell *et al.*, 2005) or phenotype by environment (Bedhomme *et al.*, 2005) interactions that could make responses to selection difficult to predict.

Another important factor that may not have been sufficiently accounted for is the tremendous diversity of strategies utilised by hosts to evade parasitism. Traditionally, studies that have attempted to identify host resistance have measured infection intensity and fitness of infected hosts under tightly controlled laboratory conditions. Whilst these studies have successfully demonstrated the fitness costs associated with parasitism and the potentially strong selective forces imposed by parasites, it is becoming apparent that they may paint unrealistic expectations of host-parasite dynamics in the field. Recent studies have demonstrated that behavioural traits, that both indirectly (Decaestecker *et al.*, 2002) and directly (Behringer *et al.*, 2006; Karvonen *et al.*, 2004) avoid sources of infection, modification to nest environments (Christe *et al.*, 2003) and mutualisms (Arnold *et al.*, 2003; Currie *et al.*, 2006) may all be important factors in determining overall levels of disease. Furthermore life-history shifts in timing of reproduction (Chadwick & Little, 2005; Krist, 2001; Minchella & Loverde, 1981) and diet alterations (Lee *et al.*, 2005) in

response to parasitism have been shown to reduce the costs of infection in a number of taxa. Taking these factors in to account it is easy to see why simple measurements of resistance in the laboratory may not shed sufficient light on the potential for parasite-mediated dynamics.

Another previously neglected factor that may hinder parasite-mediated selection is the co-occurrence of different life history strategies within a population (Hairston & Kearns, 1996; Sinervo *et al.*, 2000). For example, variation in the timing of resting egg production, and subsequent length of diapause has been shown to be important in relation to predator avoidance (Hairston & Kearns, 1996). It has also recently been suggested that resting egg production may serve as a mechanism to escape from parasites (Mitchell *et al.*, 2004). This possible link between resting egg production and parasite avoidance in natural populations raises some interesting questions regarding parasite-mediated selection. In particular, a situation might arise whereby some genotypes invest in resting egg production prior to a parasite epidemic. The offspring of such genotypes would escape the epidemic and evade parasite-mediated selection. Resting egg production may therefore inhibit the evolution of resistance among a subset of genotypes within the population.

In this thesis I investigate parasite mediated selection in a natural population of *Daphnia magna* that experiences an annual epidemic of the bacterial pathogen *Pasteuria ramosa*. I monitor host population composition and genotype frequency changes, using molecular markers, in relation to the epidemic. I also explore the effects that resting egg production may have on host-parasite dynamics, and the potential environmental cues that may trigger the onset of resting egg production.

1.3. The *Daphnia magna* - *Pasteuria ramosa,* host – parasite model system

1.3.1 The host; Daphnia magna

Daphnia are small, freshwater planktonic crustacea found in still, fresh water bodies. The *Daphnia* genus comprises more than 50 species worldwide (Hebert, 1978). The life-history and biology of these species has been well documented, and they have been the subject of numerous investigations that have examined population dynamics (Carvalho, 1987; Carvalho & Crisp, 1987; Hebert, 1978), predator-prey dynamics (Boersma *et al.*, 1998; Lass & Bittner, 2002; Slarsarczyk *et al.*, 2005), reproductive strategies (Innes *et al.*, 2000; Innes & Singleton, 2000) and host-parasite coevolution (Ebert, 2005 and references therein).

Most *Daphnia* species, reproduce by cyclical parthenogenesis, reproducing asexually for the majority of the time, with occasional bouts of sexual reproduction (Figure 1.1.). During the parthenogenetic phase, females produce clutches of 1 – 100 eggs by mitosis, usually after every adult moult. The offspring, which are usually female, are released from the brood chamber and take between 5 – 10 days to reach maturity. An adult female can produce clutches of eggs every 3 - 4 days, and can live for up to three months in the laboratory, depending on conditions.

During the sexual phase of reproduction, male offspring are produced mitotically, and both sexes produce gametes by meiosis. Sexual reproduction leads to the production of a resting egg, encased in an ephippium, which is resistant to freezing and drying, and can remain in the sediment for considerable periods of time before hatching. Sexual reproduction in *Daphnia* is triggered by environmental stimuli such as photoperiod, food shortages, temperature and fish kairomones (Carvalho & Hughes, 1983; Hobaek & Larsson, 1990; Kleiven *et al.*, 1992;

Slarsarczyk *et al.*, 2005). Photoperiod and temperature are also important determinants for the hatching of resting eggs (Caceres & Schwalbach, 2001; Stross, 1966), and field investigations have established that emergence from the resting stage largely occurs in spring (Caceres, 1998; Wolf & Carvalho, 1989). Sexual reproduction in *Daphnia* thus creates a dormant reservoir of genetic variation that periodically contributes to the population.

Daphnia life history provides a unique opportunity to unravel some of the complexities associated with studying parasite-mediated selection in natural settings. Their predominantly asexual mode of reproduction (Figure 1.1) enables clonal selection to be monitored using molecular markers in relation to parasite prevalence and epidemics (Little & Ebert, 1999; Little & Ebert, 2001; Mitchell *et al.*, 2004). The clonal aspect of their life history also enables live samples to be collected from the field and, once isolated, maintained as iso-female lines in the laboratory. That live samples collected from the field are an accurate representation of clones present in the population at the time of collection facilitates investigation in to the genetic and environmental influences of infection phenotypes in the field. This facet of *Daphnia* reproduction has been exploited, for example, as a means of showing that variation in resistance reflects variation in parasite prevalence among host genotypes in natural populations (Little & Ebert, 2000). The geographic structure of *Daphnia* populations further facilitates the investigation of parasite-mediated dynamics, since populations are defined by natural boundaries within which individuals will experience similar selection pressures. Habitats such as ponds or lakes enable consistent sampling of a population through time, and permit comparative studies between local populations (Ebert *et al.*, 1998; Haag & Ebert, 2004), as well as the comparison of individuals derived from the same and different populations (Ebert, 1994).

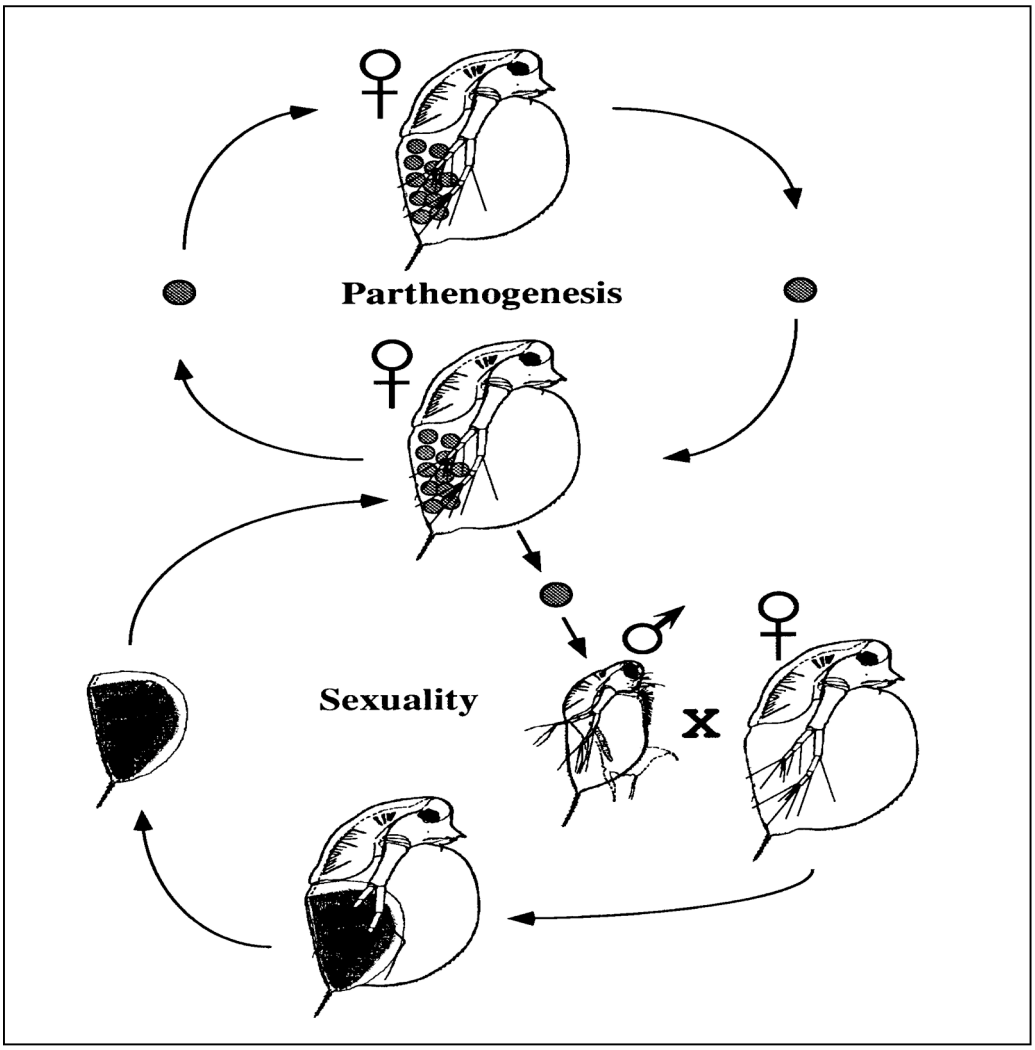

Figure 1.1. The life-cycle of a cyclically parthenogenetic *Daphnia*. *Daphnia* reproduce asexually most of the time with occasional bouts of sexual reproduction.

1.3.2. The parasite; Pasteuria ramosa

Pasteuria ramosa is a bacterial, spore forming, obligate endoparasite of *D. magna,* which is horizontally transmitted by the release of spores from decomposing cadavers of infected hosts. *Pasteuria ramosa* has a cyclical existence within populations appearing in early summer and disappearing by early winter (Green, 1974; Stirnadel & Ebert, 1997). Infection is extremely costly for *Daphnia*, often resulting in complete sterilisation (Ebert & Herre, 1996). This is reflected in measures of parasite fitness which correlates negatively with host fecundity (Ebert *et al.*, 2004). Approximately twelve days following infection, it is possible to identify the first parasite stages under a microscope (Ebert *et al.*, 1996), with the transmission stages being visible after about 20 days. Heavily infected hosts will be completely filled with transmission stages, and are red in colour, making infection easy to detect by eye. The transmission stages are easy to identify under the microscope, allowing quantification of parasite fitness using a haemocytometer. *P. ramosa* transmission spores can be stored frozen, which enables comparison of spores at different time points, and from different individuals.

1.3.3. The Daphnia magna – Pasteuria ramosa model system

The *Daphnia magna – Pasteuria ramosa* model system has been the subject of a number of investigations of host – parasite interactions in natural populations. This body of work has been a valuable contribution to current knowledge of host-parasite dynamics, and has established a model system that benefits from stable comparisons between field and laboratory. Genetic variation for resistance to *P. ramosa* has been identified in *Daphnia* populations (Little & Ebert, 1999; Little & Ebert, 2000;

Mitchell *et al.*, 2004), as have strong host genotype by parasite genotype interactions (that is, particular host genotypes are susceptible to only a subset of parasite genotypes, while particular parasite genotypes are infective to only a subset of hosts (Carius *et al.*, 2001)). Patterns of genetic change that are roughly compatible with parasite-mediated selection have been observed in natural populations (Little & Ebert, 2001; Mitchell *et al.*, 2004), and laboratory infection experiments have confirmed the genetic basis of infection patterns in the field (Little & Ebert, 2000). However, evidence for genetic change in a natural host population that is directly attributable to levels of parasite resistance is still lacking. That is, whilst genetic variation is clearly abundant, responses to selection are apparently much less observable.

In this thesis, I therefore further investigated parasite-mediated dynamics in a natural population of *D. magna,* with the particular aim to directly link field genotype frequency changes with precise measures of resistance so as to firmly test for a response to selection. I also investigated how life-history variation, specifically resting egg production may impact upon host-parasite relationships.

1.3.4. Key studies that led up to this thesis

The following *Daphnia magna* - *Pasteuria ramosa* studies are particularly relevant to this thesis. Little and Ebert (1999), Little et al (2001) and Mitchell et al (2004) all investigated host-parasite co-evolution in natural populations of *D. magna*. Little and Ebert (1999) linked clonal variation for infection in the field to changes in genotype frequencies that were consistent with parasite-mediated selection in three out of six studied populations. This was done, however, solely through the use of molecular markers and they did not confirm whether the observed changes were due to genetic differences for resistance or other factors affecting the populations. Little et al (2001)

found conflicting results in their laboratory based experiment, revealing weak genetic change in host resistance between years that were consistent with parasite mediated selection in two out of three populations but no genetic change in a third population. This result was however confusing, since previous work had confirmed that variation for infection in the field had a strong genetic basis in the third population, whereas this was not the case for one of the other two populations (Little & Ebert, 2000). Mitchell et al (2004) using molecular markers examined the genetic composition of a population before and after an epidemic within a single growing season and investigated whether genetic change observed in the field could be attributed to changes in levels of resistance then using a laboratory infection experiment. However, although the genetic composition of the *Daphnia* population differed before and after the epidemic, they could not attribute this change to higher levels of resistance for *Daphnia* collected afterwards.

Mitchell et al (2004) did, however, find support for a novel hypothesis regarding a trade-off between sexual reproduction and parasite resistance. Specifically, their laboratory infection experiment observed that *Daphnia* genotypes that were more susceptible to the parasite tended to have higher levels of resting egg production. They postulated that a situation might arise whereby some genotypes reproduce sexually in the spring prior to the annual parasite epidemic. The offspring of these genotypes would escape parasite-mediated selection, yet would survive the epidemic since resting eggs would not hatch until the following spring. In this way, the production of resting eggs may become genetically linked with higher parasite susceptibility. Both testing this hypothesis as well as testing for a response to selection generally was the major aim of this thesis.

1.4. Thesis Summary

This thesis investigates parasite-mediated selection in a natural population of *Daphnia magna* that experiences an annual epidemic of *Pasteuria ramosa*. A major theme I explore is the role of sex and diapause and how sexual reproduction may affect host - parasite co-evolution.

Chapter 2 reports on changes in population composition and population densities in a natural population of *Daphnia magna* that experiences an annual epidemic of *Pasteuria ramosa*, from April to December 2003. During this season, the population experienced an especially severe epidemic of *P. ramosa*. I collected live samples of adult females before and after the epidemic, and compared the resistance of these samples to the parasite in a laboratory infection experiment. I was also able to compare resistance levels of females that were reproducing sexually to those that were reproducing asexually, since the live sample collected before the epidemic contained a mixture of breeding types.

In Chapter 3 I examine the same host population throughout the field period studied in Chapter 2, but investigate how the parasite epidemic affected population genetic structure as indicated by molecular markers (allozymes). Patterns of allozyme variation in the field were linked to the parasite epidemic using levels of infection in a laboratory infection experiment.

In Chapter 4, I examine cues that might trigger the onset of sexual reproduction in this population. This field work showed that sexual reproduction was observed at a time when the bacterial pathogen *Pasteuria ramosa* was emerging in the *Daphnia* population, but also at a time when host population density was at its highest levels. I therefore investigate whether the presence of infected conspecifics, crowding

conditions or direct exposure to parasite spores can enhance levels of males and resting (sexual) egg production.

Chapter 2. Parasite-mediated selection and the role of sex and diapause in *Daphnia*

Duncan, A. B., Mitchell, S. E., and Little, T. J. (2006) Parasite-mediated selection and the role of sex and diapause in *Daphnia*. *Journal of Evolutionary Biology*, **19**; 1183 – 1189.

2.1. Abstract

To gain insight into parasite-mediated natural selection, I studied a natural population of the crustacean *Daphnia magna* during a severe epidemic of the bacterial parasite *Pasteuria ramosa*. I also investigated the relationship between susceptibility and the production of resting eggs which are only produced during the sexual phase of reproduction. Live host samples were taken before and after this epidemic and resistance to *P. ramosa* was examined in the laboratory. Host clones collected after the epidemic were more resistant to *P. ramosa* than were those collected pre-epidemic, which is consistent with parasite-mediated selection. In our study population, asexually reproducing females were observed across the entire study period, but females carrying resting eggs were observed only prior to the epidemic. For hosts isolated in this pre-epidemic period, I found evidence that those carrying resting eggs (at the time of collection) were more susceptible than those that were reproducing asexually. This was especially apparent for measures of parasite growth, although not all measures of infection success conclusively supported this pattern. Nevertheless, the data suggest that some genotypes invest heavily in diapause at the expense of immunocompetence. Sex could therefore inhibit the evolution of resistance because each spring new genotypes will hatch from resting eggs that are relatively susceptible as they were not exposed to the previous years bout of parasite-mediated selection.

2.2. Introduction

Parasites are thought to have extensive effects on host population genetic diversity, and may even be the selective force maintaining sexual reproduction (Haldane, 1949; Hamilton *et al.*, 1990; Jaenike, 1978). This idea has a broad theoretical basis (Bell & Maynard-Smith, 1987; Hamilton *et al.*, 1990; Otto & Nuismer, 2004; Peters & Lively, 1999) and numerous empirical studies have corroborated the evolutionary significance of parasitism. These include studies showing substantial genetic variation for infection-related traits (Henter & Via, 1995; Kraaijeveld & Godfray, 1999; Little & Ebert, 2000a), patterns of genetic variation that are compatible with frequency dependent coevolutionary dynamics (Dybdahl & Lively, 1995; Ebert, 1994; Lively & Dybdahl, 2000), and a link between breeding system and the distribution of disease prevalence (Lively, 1987; Lively & Jokela, 2002). However, neither theoretical nor empirical support for the notion that parasitism can maintain sex has been universal. For example some models indicate that the selective effects of parasites must be unrealistically severe (Howard & Lively, 1994; May & Anderson, 1983; Otto & Nuismer, 2004), and indeed the expected rapid parasite mediated dynamics have not been commonly observed in studies of natural systems (Little, 2002).

One important perspective that may require further attention is that sex often serves other functions in organisms that alternate sexual and asexual reproduction. For example sex is often associated with the production of diapausing stages that allow an organism to persist through periods of environmental hostility (Grishkan *et al.*, 2003; Hairston & Kearns, 1996; Slarsarczyk *et al.*, 2005). Parasitism is a ubiquitous source of environmental hostility so when sex leads to diapause, coevolutionary interactions between hosts and their parasites may be altered. When resting stages hatch, they could release a reservoir of genotypes that have escaped the most recent bout of

parasite mediated selection. It may therefore be difficult to disentangle the functions of sex in taxa where sex is linked to resting stages.

Daphnia are cyclical parthenogens, reproducing asexually for the majority of the year, with occasional bouts of sexual reproduction. Environmental stimuli such as photoperiod, food shortages, temperature and fish kairomones (Carvalho, 1983; Hobaek, 1990; Kleiven, 1992; Slarsarczyk, 2005) are all cues that contribute to the onset of sexual reproduction. *Daphnia* also show genetic differences for levels of sexual and asexual egg production (Deng, 1996; Hebert, 1974a). Sexual reproduction in *Daphnia* results in the production of resting eggs encased in ephippia which are resistant to freezing and drying and remain in the sediment for a period of time before hatching. Thus, sexual reproduction in *Daphnia* creates a dormant reservoir of genetic variation that periodically contributes to the population.

This study tested for the occurrence of parasite-mediated selection in a natural population of *Daphnia magna* and the bacterial pathogen *Pasteuria ramosa*. I further sought to determine whether genetic variation with regards to sexual reproduction in *Daphnia magna* could be subject to natural selection by parasites. Like many *Daphnia* parasites, *P. ramosa,* has a cyclical existence within *D. magna* populations, appearing only in summer (Stirnadel & Ebert, 1997). Previously, it was observed that *Daphnia* genotypes that are more susceptible to the parasite tend also to invest more in sexual reproduction (and hence resting eggs) (Mitchell *et al.*, 2004). This situation would arise if genotypes that produce resting eggs in the spring create a reservoir of progeny that escape the peak of parasite-mediated selection in the summer, because once made, resting eggs will often not hatch until the following spring. Thus, the production of resting eggs prior to the summer epidemic could become genetically

correlated with higher parasite susceptibility as susceptible genotypes are not removed by selection during the summer.

I monitored a natural population of *D. magna* for bouts of sexual reproduction across a season with a strong parasite epidemic, and brought clones into the laboratory to test their susceptibility. Susceptibility could be indicated by any of three response variables that I measured in the laboratory; parasite growth, the proportion of hosts becoming infected, or parasite-induced fitness losses in hosts. I studied hosts from both before and after the epidemic, and also compared those showing variation in the propensity for sex/resting egg production. Our specific predictions for the infection experiment were:

1) Hosts collected after the epidemic would be less susceptible than hosts collected before, having just experienced that summer's parasite epidemic, i.e. I predicted that the epidemic would select for resistance.

2) Within the pre-epidemic samples, hosts reproducing sexually at time of collection would be more susceptible than hosts reproducing asexually at time of collection being represented by genotypes that do not typically experience parasite-mediated selection during summer epidemics.

2.3. Materials and methods

2.3.1. Organisms and collections

D. magna is a planktonic crustacean found in still freshwater bodies and is host to numerous bacterial, microsporidian and fungal parasites (Green, 1974; Little & Ebert, 1999; Stirnadel & Ebert, 1997). *Pasteuria ramosa* is a bacterial, spore forming, obligate endoparasite of *D. magna* that greatly reduces host fecundity. Transmission

is horizontal, achieved by the release of spores from the decomposing cadavers of previously infected hosts, (Ebert *et al.*, 1996).

Daphnia magna and *P. ramosa* were collected from a farm pond at Leitholm, Scottish Borders (2°20.43'W 55°42.15'N). Samples were taken 1 - 2 times a month between April and December 2003. Three samples were taken at each collection from different locations around the pond. Variability between samples due to sampling techniques were minimised by always using the same net and sweep length.

Immediately following collection, population composition was estimated. Each sample was sieved and diluted in 250ml of water. The sample was well mixed, and sub-samples were poured on to a petri dish. Water was removed, and each sub-sample analysed under a dissecting microscope. Individual *D. magna* were recorded as follows; adult females with asexual eggs, adult females with ephippia (reproducing sexually), barren adult females, juveniles, and males. I counted until at least 100 individuals had been recorded. Prevalence of the parasite *P. ramosa* was recorded by eye across all samples. Infected *D. magna* are usually much redder in colour making infection easy to detect by eye.

Each month one live sample of adult females was kept and from these I established iso-female lines. When a relatively large portion of the population was found to be reproducing sexually (this occurred at two sampling dates; 14th May 2003 and 27th June 2003), live samples comprising females with ephippia also were kept. Since I ensure that *D. magna* only reproduce asexually in the lab, once isolated a female (regardless of whether she was reproducing asexually or sexually at time of collection) and all her subsequent offspring are a genetically identical clone. Thus, each live sample can be considered a representation of clones present in the pond at time of collection.

2.3.2. Infection experiment

An infection experiment was performed on *Daphnia* that had been collected in May and June, (before the parasite epidemic reached its peak and consisted of females reproducing sexually and asexually), and November (once the epidemic had abated, and was composed entirely of females reproducing asexually at time of collection). Thus there were a total of 96 individual iso-female lines, termed clones, that contributed to three experimental groups; 1) pre epidemic and reproducing sexually at time of collection, 2) pre epidemic and reproducing asexually at time of collection, and 3) post epidemic, all of which were reproducing asexually at time of collection. As stated in the introduction, I predicted that group 1 would be more susceptible than group 2, and that group 3 would be least susceptible of all.

To equilibrate maternal effects, three replicates of each clone were kept under experimental conditions for three generations prior to starting the experiment. Replicates contained 5 females all from the same clutch, in a 200ml jar of *Daphnia* medium (Klüttgen *et al.*, 1994). All subsequent generations of each replicate, including the experimental generation were seeded using females from the 3rd, 4th or 5th clutches that were less than 24 hours old.

A solution of *P. ramosa* transmission spores that had been frozen at -20°C was used for the infection experiment. The spores in solution originated from a large mixture of *D. magna* infected with *P. ramosa* collected from the same pond in 2000. Creating the solution involved infecting a mixture of *Daphnia* individuals (from fifteen clones taken from the same population) with *P. ramosa*. Infected individuals were frozen, eventually being crushed together to form the spore solution. Mitchell et al (2004) confirmed in a pilot study that there is no significant difference in infection rates between spores collected in different years.

The infection experiment comprised three replicate jars containing five females of each of the ninety six clones set up over 4 days. Five female offspring less than 24 hours old were placed in a jar containing 50ml of *Daphnia* medium, with purified sand at the bottom. Sand in jars during infection periods reduces variation in infection levels and increases the incidence of infection (Mitchell *et al.*, 2004). To each jar, 1×10^5 *P. ramosa* transmission spores were added. Everyday, until day 8, each jar was stirred to increase chances of contact with parasite spores. During the infection period *Daphnia* were fed 1×10^7 algae cells on day 1, and 5×10^6 algae cells on days 3 and 6. This comparatively low level of food encourages the *Daphnia* to graze the sand, increasing contact with the parasite. Throughout the experiment all *Daphnia* were kept at 20°C, and experienced a light:dark cycle of 16:8 hours.

On day 8 each group of 5 *Daphnia* were transferred to a jar containing 200ml of *Daphnia* medium and fed 1.75×10^7 algae cells per day until the end of the experiment. Each jar was checked for newborn daily. When newborn were present the adult females were moved to a new jar, and the offspring in the clutch counted. In the absence of any clutches *Daphnia* were transferred to a new jar with fresh medium every 3 days. The experiment finished on day 25 at which time each individual *D. magna* was frozen in a 1.5ml eppendorf tube. Frozen *Daphnia* were later crushed in 100µl of water, and then 8µl of this was placed on to a Nebauer haemocytometer where I could confirm infection and count *P. ramosa* transmission stages (an estimate of parasite fitness).

2.3.3. Life-history experiment

In a separate experiment I investigated whether females from the three groups differed in reproductive output in the absence of infection. A total of 86 clones contributed to the experimental groups; 1) pre epidemic and reproducing sexually at time of collection, 2) pre epidemic and reproducing asexually at time of collection, and 3) post epidemic, all of which were reproducing asexually at time of collection. Methods were identical to those described for the Infection experiment, except each clone was represented by one replicate, and the experiment finished on day 30.

2.3.4. Data analysis

I used general linear models as implemented in JMP 5.1 to investigate how parasite transmission spore production, the proportion of hosts infected, and host offspring production were affected by 'field history' in the infection experiment. Field history is a fixed factor with three levels; 1) pre-epidemic hosts, reproducing sexually, 2) pre-epidemic hosts, reproducing asexually, and 3) post-epidemic hosts (which all happened to be reproducing asexually at the time of collection). Host clone was included in each model as a random effect, nested within 'field history'. The experiment was set up over four days and thus 'set up day' was also included as a random effect. Proportion data were arcsine-square root transformed, offspring counts were square-root transformed, and transmission spore counts were log transformed. A general linear model was also used to investigate how host offspring production was affected by 'field-history' in the life-history experiment. As before offspring production was square-root transformed.

I next addressed our 2 hypotheses separately. First I compared all pre-epidemic hosts with post-epidemic hosts to test for parasite-mediated selection. I then

looked solely at hosts collected before the parasite epidemic, and compared those that were reproducing sexually at time of collection with those that were reproducing asexually to test for susceptibility differences associated with life-history.

2.4. Results

2.4.1. Population composition

Pasteuria ramosa was not present in samples collected early or late in the year, however an epidemic occurred during the summer reaching 100% prevalence in July. Peak parasite prevalence corresponded with a dramatic drop in *Daphnia* abundance (Figure 2.1). The incidence of sexual reproduction (measured as the occurrence of both males and females carrying ephippia) was highest in May and June (Figure 2.2). Barren and juvenile females consistently composed between 70% and 100% of the population and these are omitted from figure 2 as they obscure the dynamics of the reproducing portion of the population. It should be noted that these population density dynamics would have been influenced by a variety of factors such as competition for food.

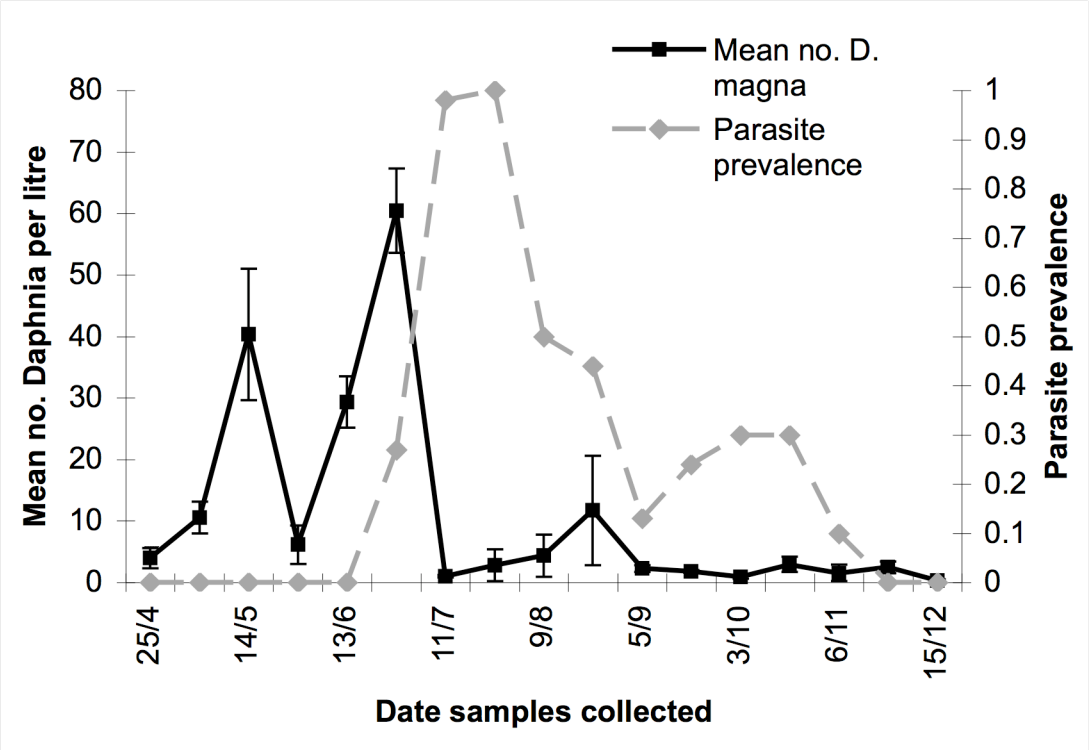

Figure 2.1: Mean number of *Daphnia* per litre collected from the Leitholm population in 2003 and proportion of population infected with *Pasteuria ramosa*. (± standard error).

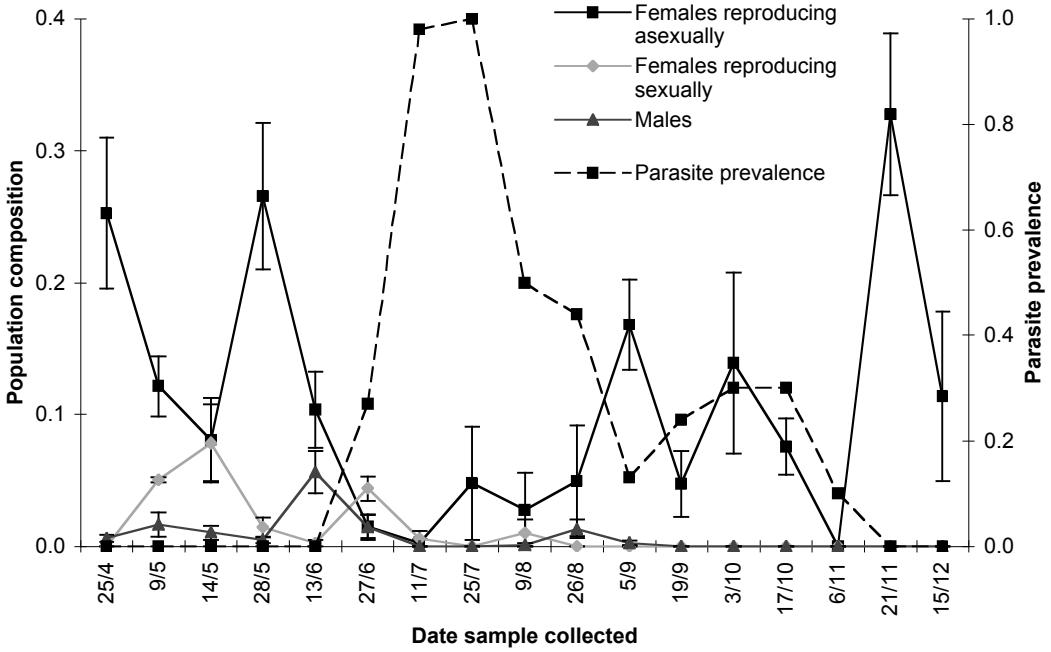

Figure 2.2: Proportion of sample composed of reproducing females and males in the Leitholm *Daphnia* population, estimated from 3 live samples collected on each date, and proportion of population infected with *Pasteuria ramosa* (± standard error).

2.4.2. Infection and life-history experiments

Parasite growth, measured as mean number of transmission spores per host, was significantly affected by field history in the predicted direction (Figure 2.3; $F_{2, 87}$ = 9.79, $P < 0.0001$). Field history also significantly impacted levels of infection among the three host groups in the predicted direction (Figure 2.4; $F_{2, 93} = 9.14$, $p < 0.0002$). Reflecting these differences in infectivity and parasite growth, field history significantly impacted offspring production among the three host groups (Figure 2.5; $F_{2, 93} = 3.37$, $p = 0.039$). It should be noted that the overall differences in offspring production when exposed to parasites is not due to intrinsic differences in the clones in the absence of infection. The life-history experiment confirmed that offspring production in the absence of parasites did not differ among the three groups (Figure 2.6; $F_{2, 77} = 0.16$, $p = 0.85$).

Regarding the prediction that the epidemic will have selected more resistant hosts, parasite growth was higher on host clones collected before the parasite epidemic than those collected after (Figure 3; $F_{1, 88} = 17.32$, $p < 0.0001$). Infection levels were also higher in hosts collected before the parasite epidemic (Figure 4; $F_{1, 94}$ = 14.85, $p < 0.0002$). Although not significant there was a trend for hosts collected after the epidemic to have more offspring in the face of parasitism (Figure 5; $F_{1, 94}$ = 3.02, $p = 0.085$).

Examining only the pre-epidemic samples, parasite growth was higher on hosts that were reproducing sexually in the field than those reproducing asexually at the same time (Figure 2.3; $F_{1, 55} = 4.23$, $p = 0.044$). Despite this, intrinsic infection levels were not found to differ between females reproducing sexually at time of collection and those reproducing asexually (Figure 2.4; $F_{1, 58} = 2.02$, $p = 0.16$), however the difference is again in the predicted direction. There was not found to be a significant

difference in offspring production between these two groups (Figure 2.5; $F_{1, 58} = 2.93$, $p = 0.092$).

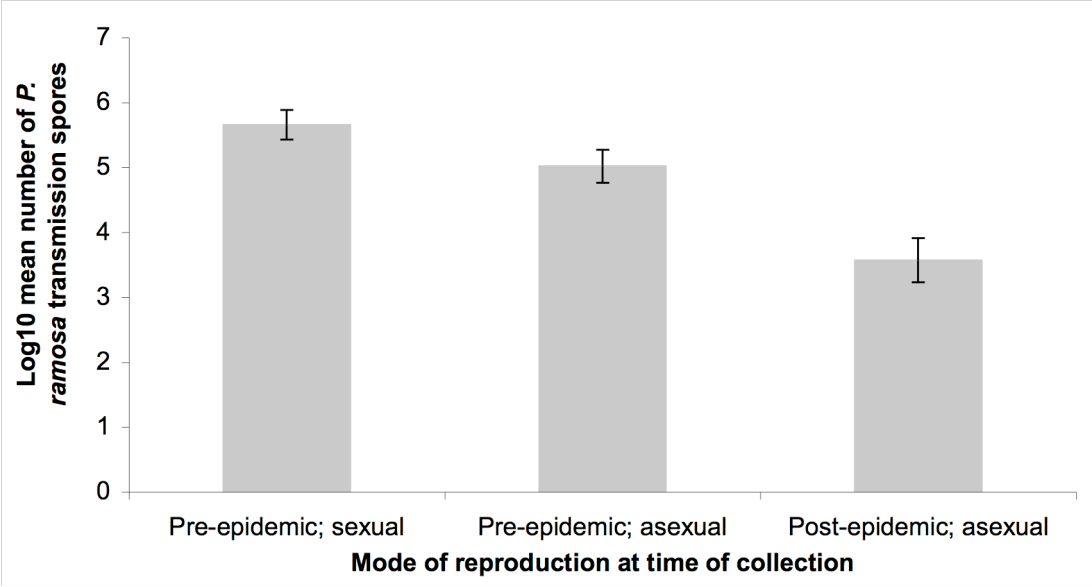

Figure 2.3: Parasite fitness measured as mean number of transmission spores per host produced across those reproducing sexually and asexually before the epidemic and asexually after the epidemic (± standard error). This figure shows transformed data.

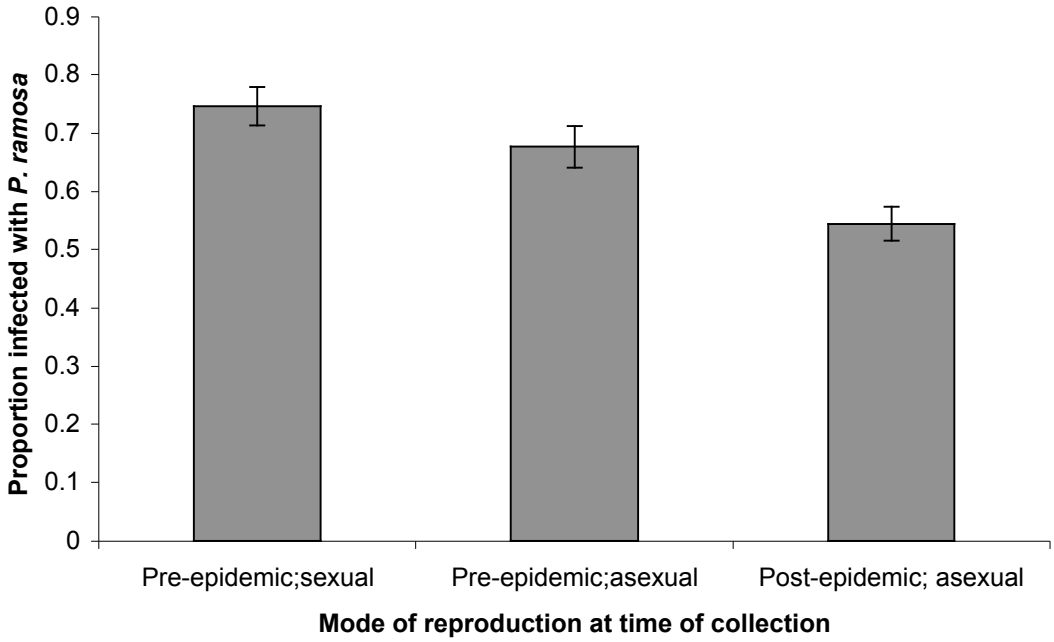

Figure 2.4*:* Resistance to *P. ramosa* among *Daphnia* collected before the epidemic reproducing sexually and asexually before the epidemic and asexually after the epidemic (± standard error). Infection inferred through direct observation of P. ramosa spores for 194 of the replicates. This figure shows original untransformed data.

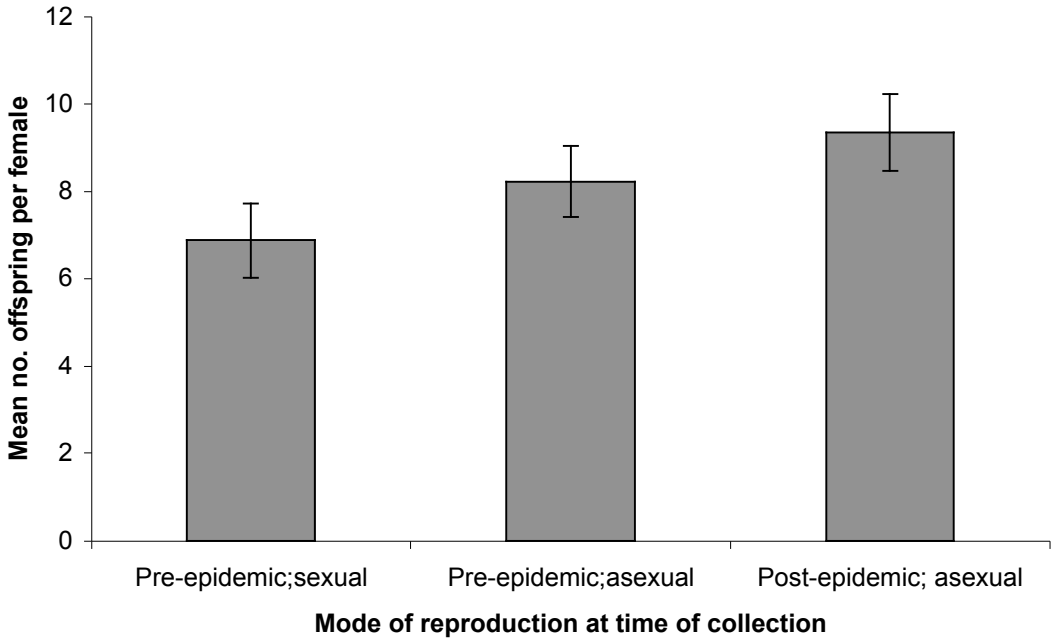

Figure 2.5: Comparisons in host fitness between *Daphnia* that were reproducing sexually and asexually before the epidemic, and asexually after the epidemic in the presence of the parasite; measured as mean number of offspring per female (± standard error). This figure shows original untransformed data.

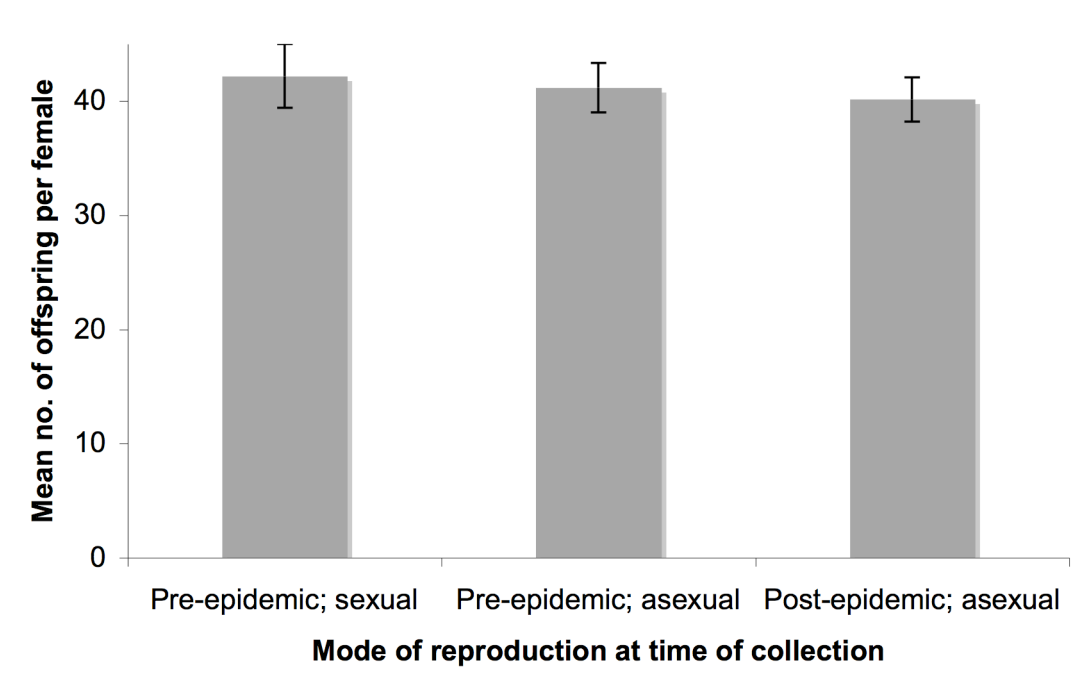

Figure 2.6: Comparisons in host fitness between *Daphnia* that were reproducing sexually and asexually before the epidemic, and asexually after the epidemic in the absence of the parasite; measured as mean number of offspring per female (± standard error). This figure shows original untransformed data.

2.5. Discussion

I observed a severe summer epidemic of the bacterium *Pasteuria ramosa,* with infection prevalence reaching 100 percent in the *Daphnia magna* host population. To test if this epidemic was a source of natural selection, I collected hosts from before and after this epidemic and found those collected before the epidemic were more susceptible to *P. ramosa* than post epidemic hosts. This pattern of susceptibility was evident as higher parasite growth and a greater proportion of hosts becoming infected in the pre-epidemic set of isolates. Thus the parasite epidemic appears to have pruned the more susceptible genotypes from the population.

This study therefore demonstrates parasite-mediated selection in a naturally interacting host-parasite system. Previous *D. magna* - *P. ramosa* studies have had variable success at finding such evidence for parasite mediated selection in the wild (Little, 2002; Little & Ebert, 1999; Little & Ebert, 2001; Mitchell *et al.*, 2004) but see (Haag & Ebert, 2004) for an example with a different parasite in an semi-natural population). Field work on our study population at Leitholm in the year 2000 (i.e. three years prior to the present study, see Mitchell et al 2004) was unable to demonstrate parasite-mediated selection in a experimental design similar to the present one.

It is notable that parasite prevalence in 2000 reached only 30%, while in 2003, the year sampled for the present study, it reached 100%. Thus the 2003 host population almost certainly experienced stronger parasite-mediated selection. This large difference in parasite prevalence and selection pressure between years could well be due to temperature. The summer of 2003 was one of the hottest on record in Europe (Schar, 2004), and *P. ramosa* shows greater infectivity and causes higher virulence at higher temperatures (Mitchell *et al.*, 2005). The high temperatures of

2003 also caused reduced pond depth which could have increased the encounter rate of *D. magna* with parasite spores which lay in the sediment.

Despite the occurrence of parasite-mediated selection within a season, there may be limits to the evolution of host resistance in the longer term. Most obviously, a subsequent evolutionary response in the parasite population would erode any gains made by the host population. In addition, this study, and a previous one, provide support for a novel hypothesis on the limits of evolution focused on the impact of recruitment from the 'seed bank' of resting eggs. In the earlier study (Mitchell et al 2004), resting egg production was induced in the laboratory and it was observed that those genotypes that tended to produce more resting eggs (in the absence of parasites) also tended to be more susceptible when exposed to parasites. In the present study, I corroborated this by showing that one of our measures of susceptibility, parasite growth, was higher on those hosts that were carrying resting eggs than on those that were reproducing asexually at the time of collection. This corroboration, however, was not complete as two additional measures of susceptibility, infection levels and host reproduction, did not fit this pattern, although the trend was in the correct direction. However, I consider these statistical tests to be conservative given that they do not incorporate the directional nature of our predictions.

Thus, a life history strategy that employs sexual reproduction prior to a parasite epidemic appears to be genetically associated with lower parasite resistance. This association will arise because one set of genotypes invests in resting eggs (that lie dormant until the following spring) prior to the summer parasite epidemic. Consequently these genotypes escape the peak of parasite mediated selection pressure for higher resistance. Simultaneously, another set of genotypes that invest less in the production of resting eggs, do not escape the epidemic, and will potentially evolve

higher resistance. Subsequently, the spring emergence from the resting egg bank of more susceptible genotypes will reduce the resistance to parasites that was gained in response to the previous season's parasite epidemic.

This potential link between susceptibility and resting egg production is at least in part genetic, i.e. a negative genetic association between resistance to parasites and sex/resting egg production. Nevertheless, I do not rule out the possibility that parasites can also directly induce sex in *Daphnia*. Slusarczyk et al (2005), for example, found *Daphnia* to have increased ephippia production in the presence of fish kairomones and sufficient light. Thus ephippia production was induced as an adaptive mechanism against the threat of fish predation. Our field observations showed two bouts of ephippia production prior to the parasite epidemic, the second occurring just as the parasite appeared in the population. It may not be a coincidence that the second bout of sex occurred at this time, and I am currently investigating how ephippia production in our sexual clones could be directly induced by parasite presence. Furthermore a direct physiological trade-off between the production of ephippia and immune function is conceivable. Ephippia are composed largely of melanin, and melanin, being the end product of the phenoloxidase cascade is also an important component of the arthropod immune system (Soderhall & Cerenius, 1998). Those genotypes that reproduce sexually early in the season may be predetermined to invest their melanin in ephippia, whereas other genotypes have melanin available for investment in immune defence. This hypothesis might be testable through environmental induction of melanin production, for example by exploiting the natural variation in degree of carapace melanisation in some *Daphnia* populations which is associated with UV protection (Hebert & Emery, 1990).

The genetic association I hypothesise is similar to one generated from a study of predation that compared behavioural traits of copepods that hatched from resting eggs collected from different depths of pond sediment. Copepods from greater depths tended to hatch later, and switch to production of resting eggs at a later date (Hairston & Kearns, 1996). Hairston and Kearns (1996) postulated that genotypes within the population have adopted one of two life history strategies regarding these traits, each having different fitness values between years depending on onset of fish predation. In years when onset of fish predation is late those genotypes that both hatch later and switch to production of resting eggs later will enjoy greater fitness advantages. However, in years when onset of fish predation is early, these later hatching genotypes will experience fitness losses due to the reduced security of offspring survival through resting eggs. In years when the onset of fish predation is early, early hatching copepods that also switch to resting egg production earlier have higher fitness.

In summary, the present study provides evidence for parasite-mediated selection in the wild. This observation may have only been possible due to the exceptionally high temperatures and levels of parasitism that occurred in the year of sampling. I also found support for the hypothesis that sexual reproduction (and hence resting egg production) prior to a parasite epidemic might be associated with susceptibility. Those genotypes that tend to make more resting stages secure survival of their offspring by avoiding summer epidemics, but their immune systems are subject to less parasite-mediated selection, the result being immune systems that permit greater parasite growth. I expect the annual emergence from the resting egg bank of these more susceptible individuals to diminish gains in mean population fitness that were caused by the previous season's parasite epidemic. Sexual

reproduction is typically associated with the production of fitter offspring due to the purging of deleterious mutations, or the creation of novel highly adaptive genotypes (Burt, 2000; Hamilton *et al.*, 1990). Our results suggest, however, that sex and resting egg production may impart a type of genetic slippage (Lynch & Deng, 1994) upon a population such that sex directly reduces population mean fitness.

Chapter 3. Parasite driven genetic change in a natural population of *Daphnia*

3.1. Abstract

A substantial body of theory indicates that parasites may mould the population genetic structure of their hosts, but few empirical studies have directly linked parasitism to genetic dynamics. I used molecular markers (allozymes) to investigate genotype frequency changes in a natural population of the crustacean *Daphnia magna* in relation to an epidemic of the bacterial pathogen *Pasteuria ramosa.* The population experienced a severe epidemic during the study period in which parasite prevalence reached 100% of the adult portion of the population. The parasite epidemic was associated with genetic change in the host population. Clonal diversity was observed to decrease as parasite prevalence increased in the population, and tests for differences in the clonal composition of the population before, during and after the epidemic indicated that significant change had occurred. A laboratory infection experiment showed that the genotype most resistant to parasite infection was also the most common following the peak of the parasite epidemic. Thus, this study, which combined field observations of genotypic change with controlled laboratory experiments, provides a compelling illustration of parasite-mediated selection in the wild.

3.2. Introduction

Parasites are predicted to have extensive effects on their host populations, driving genetic change, population density changes, and speciation. Parasite-mediated natural selection may even be the major evolutionary force determining rates of recombination in their hosts, an idea known as the Red Queen hypothesis. Genetic variation for infection-related traits, a requirement for parasite-mediated selection, is abundant in natural host populations (Little, 2002; Wolinska *et al.*, 2004; Woolhouse *et al.*, 2002). Parasite mediated selection has been demonstrated in experimental populations (Capaul & Ebert, 2003; Haag & Ebert, 2004) and in populations that have been subject to artificial selection pressures (Buckling & Rainey, 2002; Ibrahim & Barrett, 1991). Several studies on wild populations have failed to directly observe genotype frequency change due to parasitism (Henter, 1995; Little & Ebert, 2001; Mitchell *et al.*, 2004; Siemens & Roy, 2005), while others have revealed genotype frequency change that is seemingly maladaptive (Burdon & Thompson, 1995) or is at least difficult to reconcile with predictions based on patterns of genetic variation (Little & Ebert, 2001; Siemens & Roy, 2005). Thus, while many populations experience strong genotype frequency dynamics, direct links to parasitism have been tenuous, and thus the impact of parasitism on the genetic structure of natural populations remains unresolved.

The lack of observed parasite mediated dynamics in natural systems is surprising considering the ubiquity of parasites and their often strong detrimental effects. Recent work has sought to gain insight into dynamics by highlighting how environmental variation may interact with infection, and the genetic basis of infection. Environmental variation has been shown to be a major factor determining the ability

of a host or its offspring to defend against parasites (Little *et al.*, 2003; Mitchell *et al.*, 2005b; Moret & Schmid-Hempel, 2001; Robb & Forbes, 2005; Sadd *et al.*, 2005) and several studies have demonstrated strong genotype by environment interactions (Fels & Kaltz, 2006; Ferguson & Read, 2002; Mitchell *et al.*, 2005a) that could make responses to selection difficult to predict. Other studies have emphasised that traits normally used to asses genetic variation for susceptibility, such as mortality or reproduction measured under tightly controlled laboratory conditions, may provide a misleading picture because in reality it is behavioural differences that determine susceptibility (Decaestecker *et al.*, 2002; Leung *et al.*, 2001), or that these traits strongly interact with other factors such as competition (Bedhomme *et al.*, 2005) or food availability (Leung *et al.*, 2001; Singer *et al.*, 2004). Lastly, the possibility that phenotypically plastic responses to parasitism could slow the response to selection has been emphasised in a variety of taxa (Chadwick & Little, 2005; Little & Kraaijeveld, 2004; Moret & Schmid-Hempel, 2004).

This more complex view on natural host-parasite interactions does not necessarily imply that parasite mediated selection is not important, rather that it may simply be difficult to detect. One hindrance to the study of change over time is the constraints imposed by the feasible size of the common garden infection experiments that are often used to study genetic variation for resistance. This can for example, affect the possible number of time points over which parasite mediated selection may be studied. An alternative method to study parasite mediated selection is to use genetic markers, which enable the processing of a larger number of individuals. Due to their short generation time, invertebrates are often targets for the study of genetic change, but it is not typically known which immune-related genes to use for the tracking of parasite mediated dynamics. Neutral genetic markers such as

microsatellites or allozymes are potentially useful for correlating general levels of diversity with parasitism, but are not expected to be directly involved in resistance or even associated with loci that are. However, in organisms with high levels of linkage disequilibrium (e.g. clonal or highly selfing taxa), associations between neutral loci and loci under selection may occur. This provides the opportunity for intensive sampling of natural populations to reveal parasite-mediated genetic dynamics (Dybdahl & Lively, 1998; Little & Ebert, 1999).

Dramatic changes in allozyme genotype frequency over time are well documented in natural populations of *Daphnia* (Carvalho, 1987; Carvalho & Crisp, 1987; Hebert, 1974b), which are cyclically parthenogenetic and often show high levels of genotypic disequilibria. Strong associations between allozyme genotypes and infection prevalences (Little, 1999) or important life-history traits (Carvalho, 1987; Hebert, 1974a) have been revealed by field studies. However, attempts to link genotypic changes to parasite-mediated selection in *Daphnia* have generated mixed results (Little & Ebert, 2001; Mitchell *et al.*, 2004), leading previous researchers to conclude, somewhat unsatisfactorily, that unmeasured environmental variables were overwhelming the effects of parasitism. Two problems with previous studies are that associations between allozyme markers and infection in the field were not verified with controlled laboratory experiments, or that studies were conducted during periods when the impact of parasitism was relatively low. The present study analysed allozyme variation in a population of *Daphnia magna* over an eight month period that spanned a very intense epidemic of a bacterial parasite that essentially sterilises its host. We observed dramatic fluctuations in allozyme genotype frequencies and brought live samples of hosts in to the laboratory to test their susceptibility with

controlled infections. This enabled me to confirm whether parasites were indeed responsible for the observed genotypic dynamics.

3.3. Materials and methods

3.3.1. Organisms and field collections

Daphnia magna is a planktonic freshwater crustacean found in still freshwater ponds. It is host to numerous bacterial, fungal and microsporidian parasites (Green, 1974; Little & Ebert, 1999; Stirnadel, 1994; Stirnadel & Ebert, 1997). Substantial genetic variation for resistance has been observed among genotypes of *D. magna* when exposed to *Pasteuria ramosa* (Carius *et al.*, 2001), a bacterial, spore forming, obligate endoparasite that is the best-studied of the *D. magna* parasites. *Pasteuria ramosa* is horizontally transmitted by the release of spores from decomposing cadavers of infected hosts (Ebert, 1996). Infection is highly costly, causing dramatic declines in host fecundity, often resulting in complete sterilisation.

Daphnia magna were collected in 2003 from a farm pond at Leitholm, in the Scottish Borders (2°20.43'W 55°42.15'N). Samples were taken twice per month between April and September when the *Daphnia* population was large or growing, and then once per month during the colder months of October-December when the population was experiencing little change. Three samples were taken at each collection from different locations around the pond, although the same three locations were always sampled. Variability between samples due to sampling techniques were minimised by always using the same net (opening of 630 cm^2) and sweep length. After each collection live samples were taken back to the laboratory where an estimate of prevalence of the parasite *P. ramosa* was checked in the adult portion of all subsamples. Infected adult *D. magna* are usually distinct making infection easy to detect by eye. Random samples of the host population were frozen in eppendorf tubes at -80°C for later allozyme electrophoresis.

I genotyped an average of 107 host individuals from each of 15 time-points using standard methods of cellulose acetate allozyme electrophoresis (Hebert & Beaton, 1993). The enzymes studied were mannose-6-phosphate isomerase (MPI), aspartate amino transferase (AAT) and fumerate hydratase (FUM), each of which had just two alleles, and all of which were known to be polymorphic based on a previous study of this population (Mitchell et al 2005). Allozyme bands with unique electrophoretic mobility were assumed to correspond to unique alleles. Accordingly individuals sharing the same electrophoretic phenotype were regarded as having the same 'electrophoretic genotype'. However, it is probable that individuals indistinguishable at these loci may differ at other loci not assayed, or possess amino acid substitutions that do not result in detectable mobility differences. This caveat applies to all allozyme studies and a substantial proportion of studies using other molecular markers.

3.3.2. Infection experiment

Live samples from before (30 individuals from 14th May 2003, 30 individuals from 27th June) and after (36 individuals from 21st November 2003) the parasite epidemic were isolated and then maintained clonally as isofemale lines in the laboratory for use in later experimentation (Duncan et al, 2006). Using methods identical to those described above, I allozyme genotyped clonal copies of these live *Daphnia*. I then performed an infection experiment on these host lines to test for susceptibility differences between electrophoretic genotypes under controlled conditions.

The experimental infection protocols are described in Duncan et al (2006). Briefly, to equilibrate environmental variation among the lines prior to

experimentation effects, three replicates of each iso-female line were kept under experimental conditions for three generations. Replicates contained 5 females all from the same clutch in a 200ml jar of *Daphnia* medium (Klüttgen *et al.*, 1994). A suspension of *P. ramosa* transmission spores that had been frozen at -20°C was used for the infection experiment. The spores in suspension originated from a large mixture of *D. magna* infected with *P. ramosa* collected from the same pond in 2000 (Mitchell et al 2004). Creating the solution involved infecting a mixture of *Daphnia* individuals (from fifteen clones taken from the same population) with *P. ramosa*. Infected individuals were frozen, eventually being crushed together to form the spore solution. Mitchell et al (2004) confirmed in a pilot study that there is no significant difference in infection rates between spores collected in different years, and consistent with this Little and Ebert (2001) found no difference in the infective properties of mixed spore solutions applied to diverse host collections.

From each replicate, five female offspring less than 24 hours old were placed in a jar containing 50ml of *Daphnia* medium, with purified sand at the bottom. The infection experiment was set up over 4 days. To each jar, 1×10^5 *P. ramosa* transmission spores were added. Everyday, until day 8, each jar was stirred with a glass rod to increase chances of contact with parasite spores. During the infection period *Daphnia* were fed 1×10^7 algae cells per jar on day 1, and 5×10^6 algae cells on days 3 and 6. This comparatively low level of food encourages the *Daphnia* to graze the sand, increasing contact with the parasite. Throughout the experiment all *Daphnia* were kept at 20°C, and experienced a light:dark cycle of 16:8 hours.

On day 8 each group of five *Daphnia* were transferred to a jar containing 200ml of *Daphnia* medium and fed 1.75×10^7 algae cells per day until the end of the experiment. Each jar was checked for newborn daily. When newborn were present the

adult females were moved to a new jar. In the absence of any clutches *Daphnia* were transferred to a new jar with fresh medium every three days. The experiment finished on day 25 at which time each individual *D. magna* was frozen in a 1.5ml eppendorf tube. Frozen *Daphnia* were later crushed in 100μl of water, and then 8μl of this was placed on to a Nebauer haemocytometer where I could confirm infection.

3.3.3. Analysis

For analysis I classified our field data into three sampling periods: before, during and after the epidemic. The parasite epidemic was considered to be the period when prevalence was greater than 0.1, thus the epidemic spans 13th June 2003 to the 17th October 2003. Differences in the frequencies of the different multi-locus electrophoretic genotypes collected before (the period 25th April to 13th June), during (within the period 27th June to 17th October) and after (the period from 6th November) the parasite epidemic were analysed using contingency table analysis (JMP). Fifteen 'electrophoretic genotypes' were identified throughout the study period, but ten that were present in low numbers were pooled into a separate 'rare' group. Criteria for the rare group entailed those genotypes that had a count less than 5 in the contingency table analysis in either the before, during or after category of the epidemic. Similarly, I investigated allele frequency change over time.

Clonal diversity at each collection date was estimated using Simpsons diversity index, corrected for sample size (Rosenzweig, 1997). Samples collected on the 25th July 2003 and 15th December 2003 were excluded as less than 10 daphnia were sampled on these dates. All 15 detected electrophoretic genotypes were used for this estimate of diversity. Each estimate of diversity was subtracted from 1 to obtain a value that increased with increasing diversity.

Conformance to Hardy–Weinberg equilibrium was determined at each locus for each sampling date also using chi-square analysis. Samples collected on the 27th July and 15th December were excluded from this analysis as too few *Daphnia* were collected on these dates. Similarly samples which had expected values less than 5, and dates when the frequency of the most common allele was greater than 95%, were also excluded from this analysis. I used analysis of variance to see if there was a difference in observed and expected heterozygosities before, during and after the parasite epidemic. Expected heterozygosities were calculated at each of the three loci using expectations of the Hardy-Weinberg equation.

For the experimental data, I used analysis of variance to study the proportion of each genotype that became infected, offspring production and parasite transmission spore production. Proportion data were arcsine-square root transformed, offspring counts were square-root transformed, and transmission spore counts were log transformed to meet the assumptions of ANOVA. In this analysis I kept the group of rare genotypes the same, again due to the low numbers of individuals. To relate genotype frequency changes in the field to susceptibility in the laboratory I calculated the percent change in frequency of each of the different genotypes from the beginning of the epidemic to the end. I then performed a Spearmans Rank correlation to relate this change in frequency to mean proportion infected, mean offspring production and mean spore production of each of these genotypes in the laboratory infection experiment All analyses were done using JMP 5.1.

3.4. Results

3.4.1. Allozyme variation in the field

Pasteuria ramosa first appeared in the population in late June, briefly reached 100% prevalence in the adult portion of the population in late July, and then declined until it was absent from the population by late November (Figure 3.1). Figure 1 shows the peak of the parasite epidemic to coincide with a dramatic drop in *Daphnia* abundance. Clonal diversity ranged over time from 0.46 to 0.82 with a mean value of 0.66. Clonal diversity declined as parasite prevalence increased in the population (Figure 3.2). However, as the epidemic abated, clonal diversity increased once again to pre-epidemic levels. Contingency table analysis to test for heterogeneity in the composition of electrophoretic genotypes collected before, during and after the parasite epidemic indicated strong genetic change over time ($\chi^2 = 141.25$, df = 8, $p < 0.0001$; Figure 3.3). Allele frequencies were found to change at loci AAT ($\chi^2 = 56.77$, df = 2, $p < 0.001$) and FUM ($\chi^2 = 12.82$, df = 2, $p = 0.0016$) before, during and after the parasite epidemic. Allele frequencies were not observed to differ significantly throughout the study period at locus MPI ($\chi^2 = 2.27$, df = 2, $p = 0.32$) (Figure 3.4).

Deviations from Hardy-Weinberg were consistently observed at locus MPI, frequently observed at locus FUM and only once observed at locus AAT (Figure 3.5). Neither observed heterozygosity ($F_{2\ 10} = 1.36$, $p = 0.30$) nor expected heterozygosity ($F_{2,\ 10} = 2.93$, $p = 0.10$) was found to change significantly before, during or after the parasite epidemic (Figure 3.5).

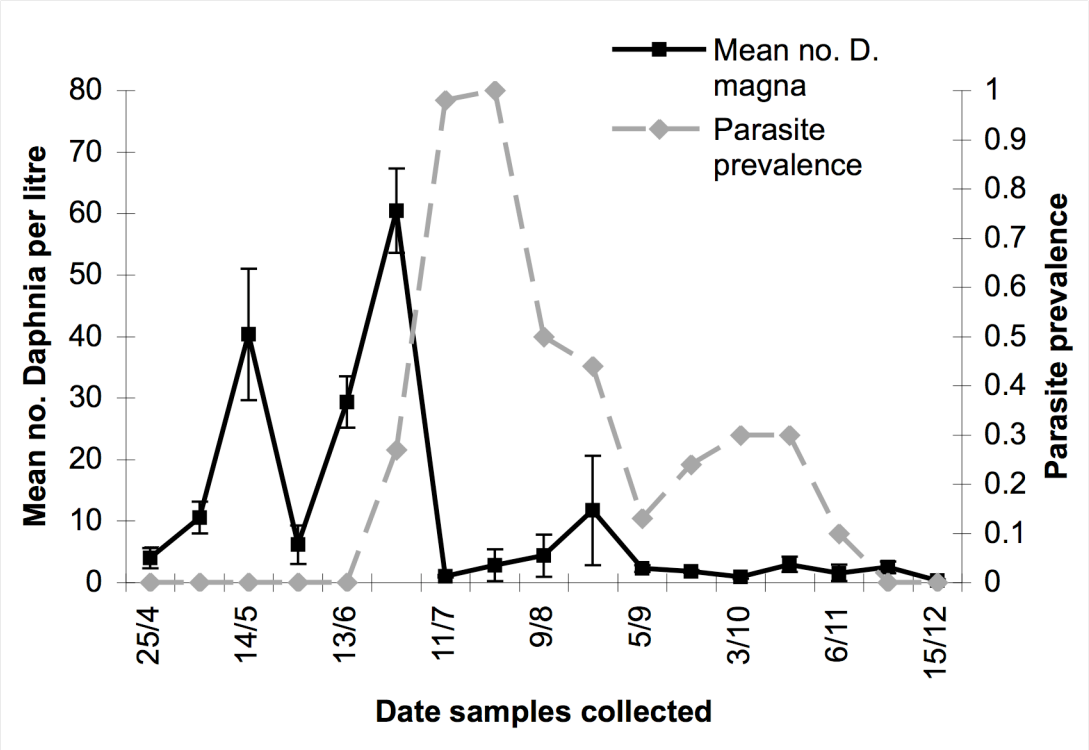

Figure 3.1: Mean number of *Daphnia* per litre and proportion of population infected with *Pasteuria ramosa*. (± standard error) in collections from the Leitholm population in 2003.

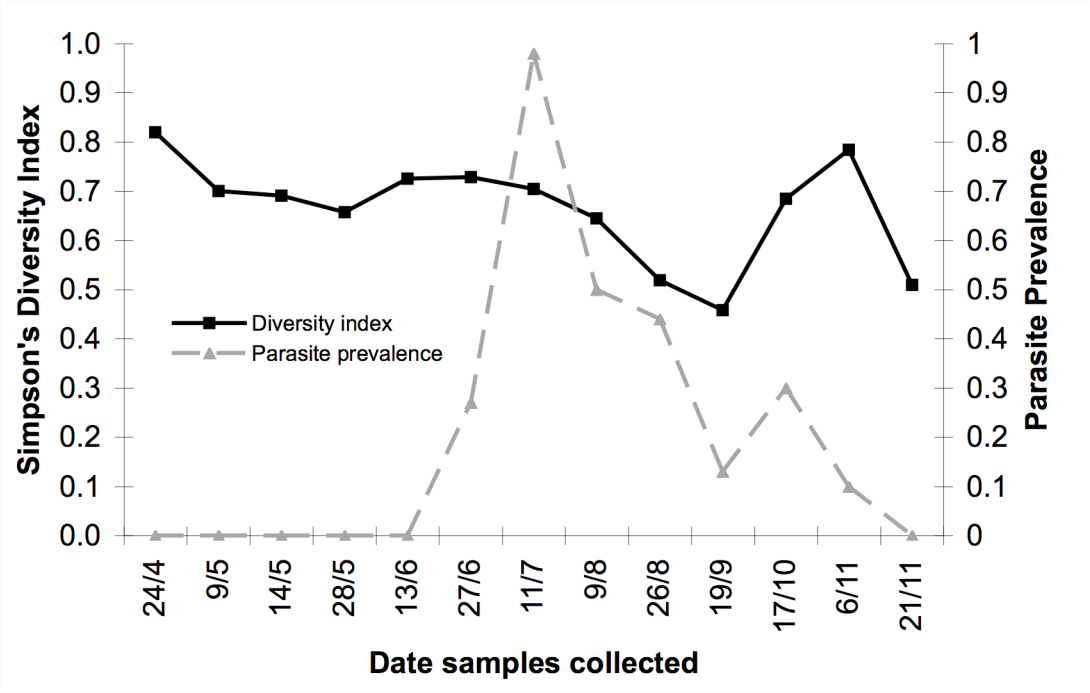

Figure 3.2: Clonal diversity, based on Simpsons Diversity Index, in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* in 2003. (Samples collected on the 25.th July and 15th December are excluded from Figure 2).

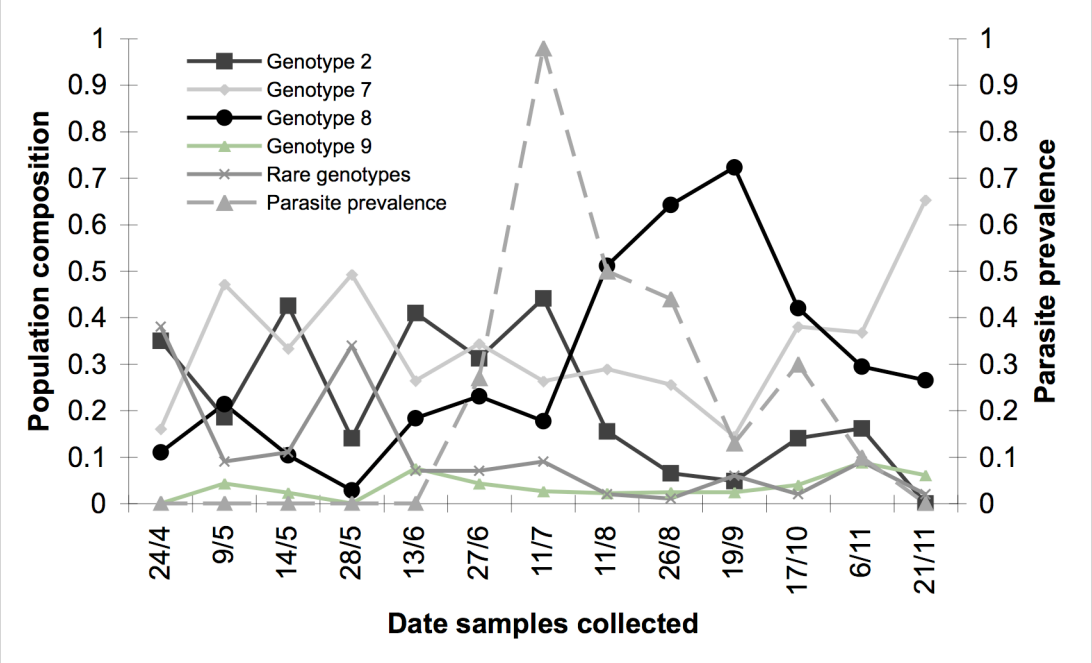

Figure 3.3: Genotype frequency changes in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* on 2003. (Samples collected on the 25th July and 15th December are excluded from Figure 3).

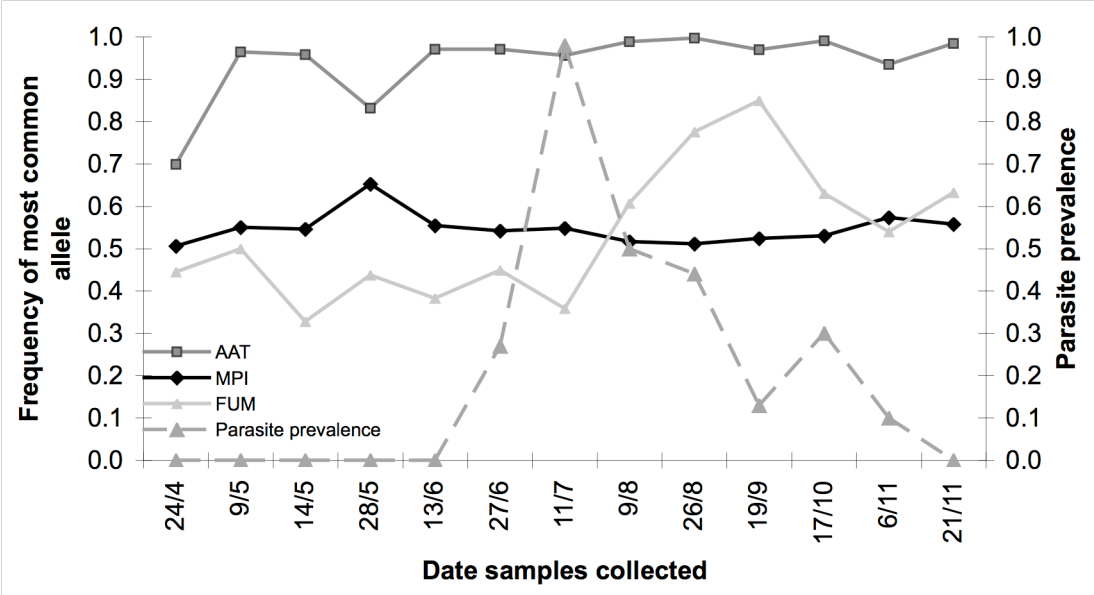

Figure 3.4: The frequency of the more common allele at loci AAT, MPI and FUM in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* in 2003. (Samples collected on the 25th July and 15th December are excluded from Figure 4).

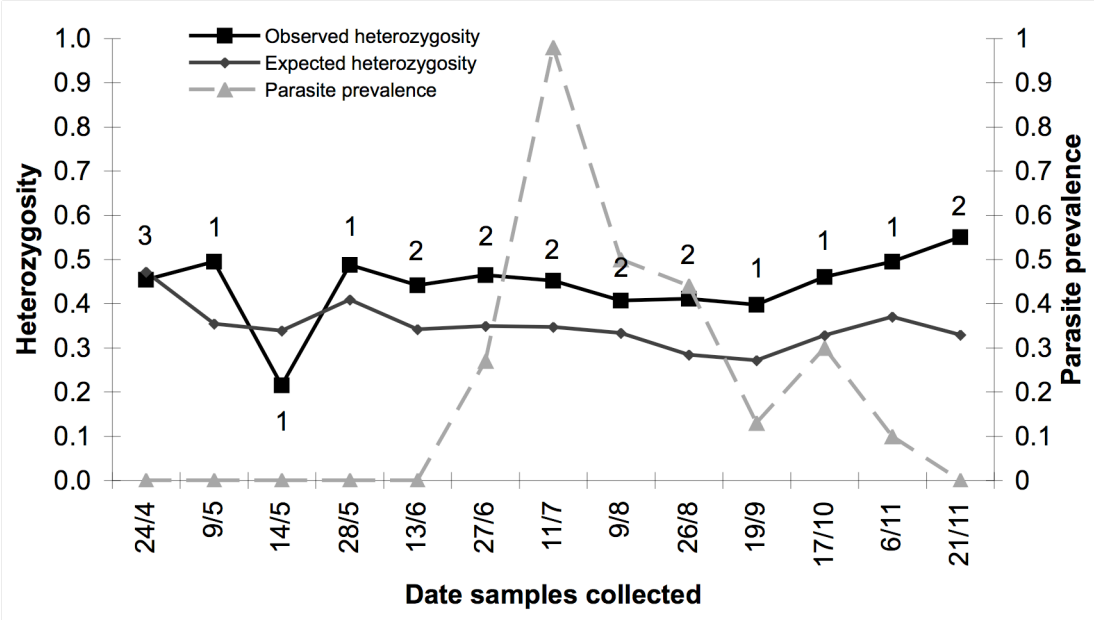

Figure 3.5: Comparison of observed heterozygosity to expected heterozygosity (± standard error) over time in the Leitholm *Daphnia* population in 2003. The numbers above the points depicting observed heterozygosity indicate number of loci where deviations from Hardy-Weinberg were detected.

3.4.2. Experimental infections

I attempted to link the parasite epidemic to genotype changes observed in the field using a controlled infection experiment. Parasite growth, measured as mean number of transmission spores per host, was found to differ significantly on the different allozyme genotypes ($F_{4, 86} = 6.09$, $p = 0.0002$). The different allozyme genotypes did not however differ in offspring production ($F_{4, 86} = 0.83$, $p = 0.51$) or levels of infection ($F_{4, 86} = 1.64$, $p = 0.17$). There was a perfect match in the ranking for each electrophoretic genotype, in terms of percent change in frequency over the course of the epidemic, and mean proportion that became infected in the laboratory infection experiment (Fig 3.6: $r = -1$, $p < 0.01$; see Neave and Worthington (1988) for discussion of significance levels when $n > 4$, and rankings are identical, or identical in the reverse). There was no relationship between percentage change in frequency during this period and offspring production ($r = 0.00$, $p = 1.00$) or parasite growth ($r = -0.3$, $p = 0.62$).

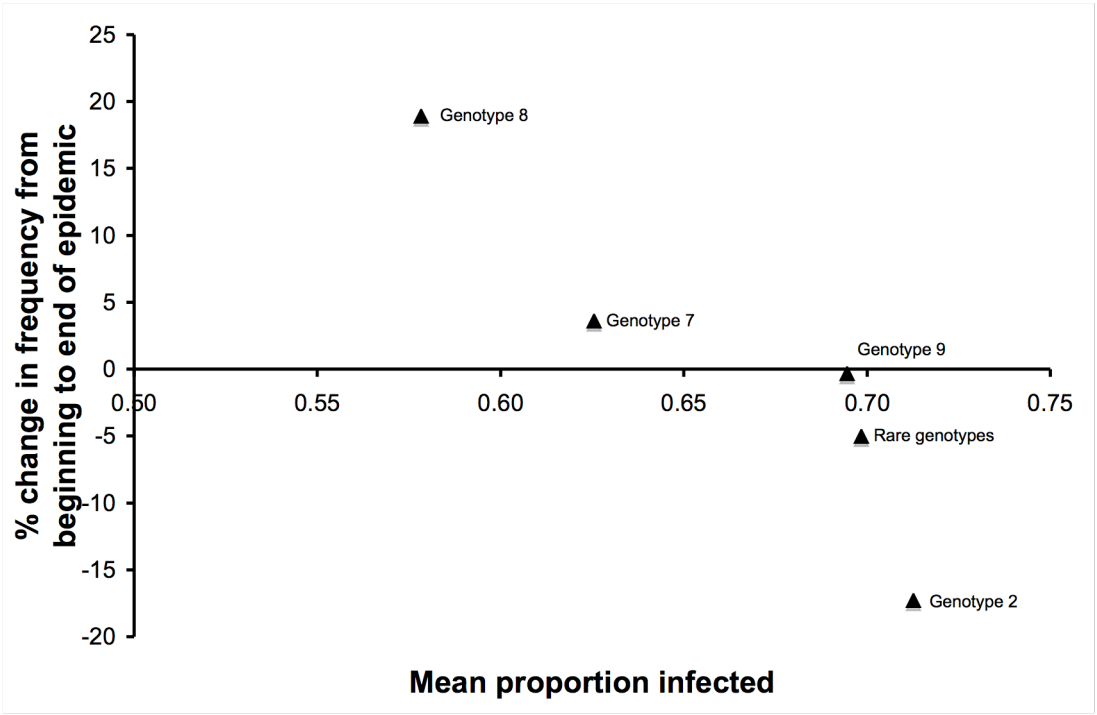

Figure 3.6: Percentage change in frequency for each of the electrophoretic genotypes from the beginning of the parasite epidemic to the end plotted against their mean levels of infection.

3.5. Discussion

This study showed that a natural and severe parasite epidemic was associated with dramatic genotype frequency (based on allozymes) changes in the host population. A controlled laboratory infection experiment revealed that the degree of decline experienced by particular allozyme genotypes was indeed related to susceptibility. This study therefore offers strong evidence of parasite mediated natural selection in the wild.

A previous study on this population (Duncan et al, 2006), also conducted on the 2003 samples, corroborates the finding of parasite mediated selection in this population. This earlier study simply compared a suite of isolates (which had not been genotyped with any molecular technique) collected before and after the epidemic and showed a decrease in average population susceptibility following the epidemic. Earlier work (Duncan et al 2006; Mitchell et al 2005), however, also indicated a mechanism that would limit the effectiveness of selection. In particular, there was a genetic correlation between the tendency to make resting eggs (which, in *D. magna,* are always the product of sexual reproduction) and susceptibility, i.e. those genetic backgrounds that tend to engage in sex also tend to be more vulnerable to parasites. This observation (Duncan et al, 2006) implied that genotypes which invest in the production of resting eggs will escape the parasite epidemic. The annual recruitment of genotypes from the resting egg bank will contribute relatively susceptible genotypes to the population and thus foster the maintenance of genetic diversity into the host population.

The present study indicates further mechanisms for the maintenance of genetic diversity in this population. Of particular interest is the observation that the genotype which increased most in frequency during the parasite epidemic (and had the lowest

levels of infection under controlled conditions; genotype 8, Figure 6), was prevalent prior to the epidemic at levels lower than the other genotypes and returned to low levels when the epidemic abated. This observation is coherent with there being a cost of resistance, although past efforts testing for costs of resistance in other *Daphnia* populations have not demonstrated them. However, the patterns of clonal dynamics observed in the present study are compatible with a number of hypotheses. For example, it is not inconceivable that this population experiences immigration that influences both allozyme genotype frequencies and mean resistance. Alternatively, genotype 8, the genotype that was most successful during the epidemic, could perform better at the warmer temperatures that coincided with the epidemic. Importantly, the cost of resistance and other hypotheses are testable in the laboratory using competition experiments.

Deviations from Hardy-Weinberg and multi-locus genotypic equilibrium are common in populations of organisms with clonal reproduction and may indicate the occurrence of selection (Hebert, 1974a, 1974b). In the present study, however, significant deviations from genetic equilibria did not coincide with parasite associated changes in genotype frequencies. Indeed, disequilibria were detectable even early in the field season. This indicates that this population is possibly not re-founded each year solely from the resting egg bank (resting eggs are produced sexually and their hatching tends to shift populations back towards genetic equilibria), but instead may harbour females that survive the winter in a parthenogenetic state. Higher levels of genetic disequilibria are expected in populations that do not experience yearly extinction due to freezing or drying, and indeed I estimate that this <1m deep pond may remain unfrozen throughout the winter. Neither observed, nor expected heterozygosities changed before, during or after the parasite epidemic. Figure 4 does

however show that observed heterozygosity was higher than expected heterozygosity indicating that deviations from Hardy-Weinberg in this population are due to an excess of heterozygotes.

Nevertheless, the parasite epidemic was associated with genetic change in the host population and laboratory experimentation supported the hypothesis that parasites caused the observed genetic fluctuations. This apparent response to selection is, as far as I are aware, among the clearest examples of direct observation of parasite-driven dynamics. Such observations appear to be rare in natural populations, which could indicate that parasite-mediated dynamics are not as substantial as required by theory on the evolutionary significance of biological interactions (Anderson & May, 1982; Howard & Lively, 1994; Otto & Nuismer, 2004; Peters & Lively, 1999). Our capacity to detect selection presently could be due to how parasite mediated dynamics may interact with environmental factors. I conducted our field work for this study in 2003, which was the year Europe experienced the hottest heat wave on record (Schar, 2004). *Pasteuria ramosa* shows greater infectivity and causes higher virulence at higher temperatures in the laboratory (Mitchell *et al.*, 2005). While epidemics are observed each summer in our study pond (Mitchell *et al.*, 2004), prevalence in 2003 was at least twice as high as in any previous year. The high temperatures of 2003 also caused reduced pond depth which could have increased the encounter rate of *D. magna* with parasite spores which lay in the sediment. Thus, our observation of parasite mediated selection in the wild is probably linked in part to environmental conditions that were conducive to an exceptionally severe epidemic.

Chapter 4. Do Parasites Induce Sexual Reproduction in *Daphnia magna*?

4.1. Abstract

Sexual reproduction in some taxa leads to the production of a resting egg that enables populations to escape unfavourable conditions. Earlier work, described in Chapter 2, showed that sexual reproduction in *Daphnia magna* may result in some genotypes, those that make resting eggs prior to the parasite epidemic, escaping parasite-mediated selection. It is not however clear what cue causes these genotypes to switch to sexual reproduction. At the time these genotypes were collected from the field, parasite prevalence was increasing, but this was also at a time when *Daphnia* population density was particularly high (Chapter 2). To investigate the cues for sexual reproduction and resting egg production I explored whether crowding conditions, which would be experienced at high population densities, or parasite infection presence, simulated by creating crowding conditions using infected conspecifics, could induce sexual reproduction in *Daphnia*. I also examined whether direct exposure to parasite spores might increase levels of sexual reproduction. Crowding conditions and cues that indicate parasite presence increased levels of male production, which is one measure of sexual reproduction. However, only crowding conditions increased resting egg production, another indicator of sexual reproduction. A water treatment type by host genotype interaction revealed that some host genotypes increase male production when in crowding conditions, but not in water indicating parasite presence, while the reverse is true for other genotypes. There was no evidence that the presence of parasite spores increased levels of sexual reproduction.

4.2. Introduction

Theory predicts that the most successful parasites are those adapted to infect the most common genotypes in their host populations. Sex, through recombination, may then combat parasites through the creation of novel host genotypes to which the parasite is not yet adapted (Haldane, 1949; Hamilton *et al.*, 1990; Jaenike, 1978; Peters & Lively, 1999). However, in some taxa, reproductive mode is linked to the production of resting stages, and of particular interest are cases such as *Daphnia* where it is the sexual phase that leads to diapause. Resting stages enable populations to avoid a range of unfavourable environments (Grishkan *et al.*, 2003; Hairston & Kearns, 1996). Parasitism is a ubiquitous source of environmental hostility and if the presence of parasites can induce sexual reproduction, then in organisms where sex is linked to diapause, sex may serve as a mechanism to avoid, rather than combat, parasites.

Although resting eggs contain novel genotypes, they will not immediately contribute to the active portion of the population. However, they are predicted to be important in maintaining genetic diversity, because when they hatch they replace genetic diversity that may previously have been lost to selection (Berg, 2005; Gomez & Carvalho, 2000). Specifically genotypes that are lost from the population can be recruited from the resting egg bank to re-establish levels of genotypic diversity present before selection occurred. If sex serves as a mechanism to avoid parasites in this way, those genotypes that emerge from the resting egg bank will not have experienced selection for greater resistance. Selection may instead favour genotypes that detect cues associated with parasite epidemics, and subsequently enter diapause. This will have implications for parasite-mediated dynamics in natural populations, and will affect host-parasite relationships in the field. For example, an annual

emergence of susceptible genotypes will counteract any increase in population mean resistance resulting from the previous season's parasite epidemic.

Duncan et al (2006) (Chapter 2) found evidence to suggest that resting egg production serves as a parasite avoidance mechanism in *Daphnia magna*. Sexual reproduction (as indicated by resting egg production) was observed in a natural population, before the annual parasite epidemic. A laboratory infection experiment subsequently established that parasite growth was higher on these hosts, indicating that these were more susceptible genotypes. Sexual reproduction was observed when prevalence of the bacterial pathogen *Pasteuria ramosa* was emerging in the *Daphnia* population, but also at a time when host population density was at its highest levels. Whether *Daphnia* respond directly to the presence of infected conspecifics in the population, or to cues that correlate with its occurrence (such as crowding) is not clear. If *Daphnia* are responding to environmental stimuli other than the presence of parasites then escaping the parasite epidemic would be incidental, although it would still limit parasite-mediated selection on genotypes that switch to diapause prior to the epidemic.

This study explores whether crowding conditions, which would be experienced at high population densities, or the presence of infected individuals, simulated by creating crowding conditions using infected conspecifics, may induce sexual reproduction in *Daphnia*. I also investigate whether direct exposure to parasite spores can increase levels of sexual reproduction in *Daphnia*.

4.3. Materials and methods

4.3.1. Organisms and field collections

Daphnia magna used in this experiment were those studied in Chapters 2 and 3, but in this chapter we limit study to those that were reproducing sexually at time of collection, as indicated by the presence of resting eggs.

4.3.2. Main Experiment

Daphnia were exposed to one of four water treatment types indicating; 1) crowding conditions, 2) presence of infected conspecifics, 3) parasite spores and 4) normal water (control). Treatment water that simulated crowding conditions was prepared by keeping *Daphnia* in 1.5 litre jars at a density of 40 *Daphnia* per litre. Offspring were removed daily to maintain a constant number of individuals contributing to the crowding environment, and each female was fed 3.5×10^6 algae cells per day. Water from these jars was collected every three days, and the *Daphnia* placed in clean water. After collection the water was filtered through a 0.2 μm Wattman filter to remove debris, and was stored in dark tanks for up to a week, until needed for the experiment. The same protocol was used to make water that indicated the presence of infected conspecifics, only I infected the *Daphnia*. Infections were achieved using standard methods similar to those described in Chapter 2, and the mean proportion of *Daphnia* that became infected in these jars was 0.63% (± 0.03 standard error).

To equilibrate maternal effects, replicates of each clone were kept in experimental conditions for three generations prior to the experiment. Replicates contained five females all from the same clutch, in a 200 mL jar of *Daphnia* medium (Klüttgen *et al.*, 1994). Each generation of each replicate, including the experimental

generations, were seeded using females < 24 hours old from the third, fourth or fifth clutches. All replicates in the experiment, including the maternal generations, were randomly assigned to a tray containing 12 replicates. The positions of replicates within each tray, as well as each tray, were moved systematically within the incubator daily.

Seven replicates of seven genotypes were used in this experiment. From each replicate, groups of five offspring (always from the same clutch) were randomly assigned to each of the four water treatments indicating either; 1) presence of infected conspecifics, 2) crowding-conditions, 3) parasite spores and 4) control. On day 1 hosts were placed in a 200mL jar containing the appropriate type of water. The water was then changed on day 5, and then on alternate days after observation of the first clutch. Every time the water was changed, each group of five females was moved to a clean jar containing the appropriate type of water. *Daphnia* that were continually exposed to parasite spores received 1×10^4 parasite spores at every water change. This low (too low to lead to infection) spore dose was chosen so that the effects of infection would not inhibit reproduction.

At each water change any offspring present were sexed and counted and the presence of resting eggs recorded. All treatment groups were exposed to a variable food regime that increases both male and resting egg production. From day one, each *Daphnia* was fed 3.5×10^6 algal cells per day, until the first clutch of offspring was observed. After this point, the food level was reduced to 1.5×10^6 algal cells per *Daphnia* per day. A change in food levels such as this is likely to enhance levels of sexual reproduction (see appendix A1). All replicates were kept at 20°C, and subjected to a 16 hour: 8 hour, light: dark photoperiod. Jars were stirred daily, and the

experiment lasted for 30 days. Appendix 1 outlines 3 experiments which provided guidance on the treatment groups and conditions applied in this experiment.

4.3.3. Analysis

Male production and resting egg production were used as measures of sexual reproduction. Analysis of variance was used to investigate whether the mean number of male offspring per female, the mean number of female offspring per female and the mean number of resting eggs produced per female were explained by water treatment and *Daphnia* genotype. All response variables were square-root transformed to conform to the assumptions of ANOVA. All analysis was done using JMP 5.1.

4.4. Results

Water treatment had a significant effect on male production, with females experiencing cues indicating crowding conditions, or presence of infected conspecifics, producing more males than *Daphnia* exposed to parasite spores, or in normal water (Fig 4.1; $F_{3, 163} = 4.44$, $p = 0.005$). There was also an effect of genotype on levels of male production ($F_{6, 163} = 7.51$, $p < 0.0001$), and an interaction between host genotype and water treatment type (Fig 4.2; $F_{18, 163} = 1.88$, $p = 0.02$), indicating that different host genotypes responded to water treatment types with different levels of male production.

Water treatment type also had a significant effect on resting egg production, with *Daphnia* experiencing cues indicating crowding conditions, or the presence of infected conspecifics, producing more resting eggs than in any of the other water treatments (Fig 4.3; $F_{3, 163} = 35.67$, $p < 0.0001$). There was also a significant effect of host genotype on levels of resting egg production ($F_{6, 163} = 46.04$, $p < 0.0001$) and a significant interaction between genotype and water treatment type (Fig 4.4; $F_{18, 163} = 2.70$, $p = 0.0005$), indicating that host genotypes differed in the levels of resting egg production in different water treatment types. Levels of female offspring production were not affected by water treatment type (Fig 4.5; $F_{3, 163} = 1.25$, $p = 0.29$), There was a significant effect of host genotype ($F_{6, 163} = 3.91$, $p = 0.0011$) but no interaction between genotype and treatment ($F_{18, 163} = 1.33$, $p = 0.18$).

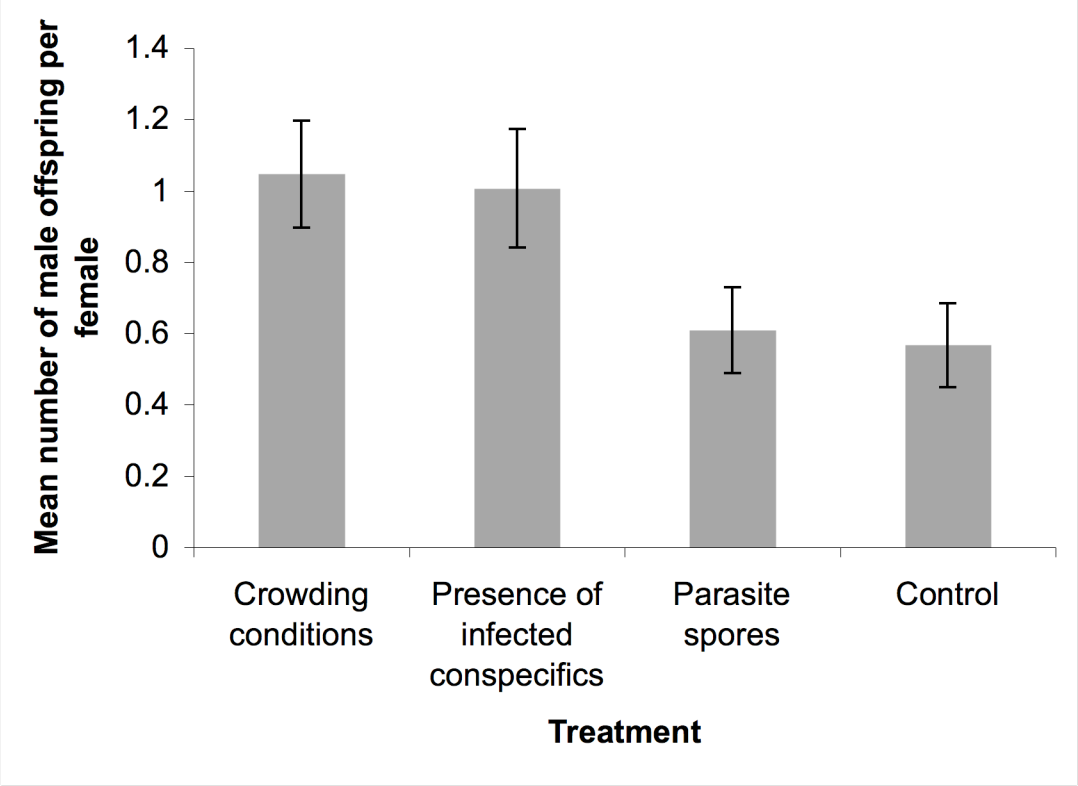

Figure 4.1. Mean levels of male production per female for *Daphnia* exposed to crowding conditions, conditions indicating the presence of infected conspecifics, parasite spores, and normal water (± standard error). This figure shows the original untransformed data.

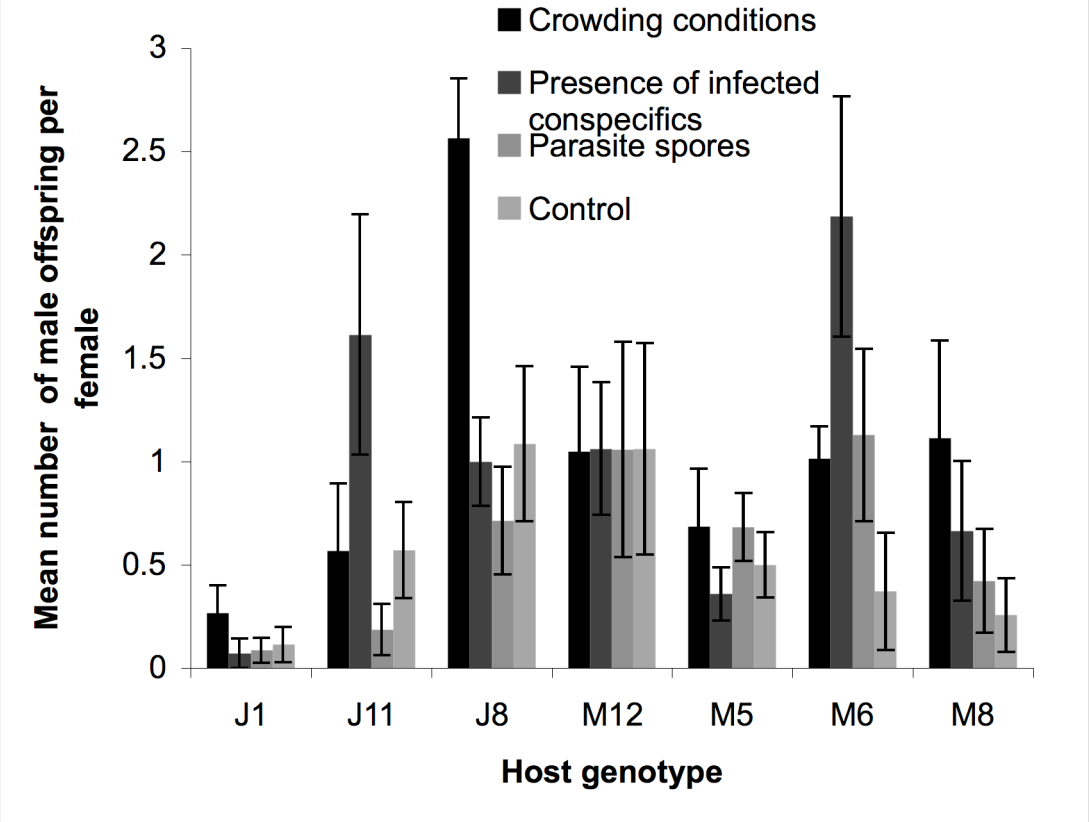

Figure 4.2. Mean levels of male production by different *Daphnia* genotypes in different water treatments (± standard error). This figure shows original untransformed data. This graph shows that genotypes J11 and M6 have increased levels of male production in response to cues indicating the presence of infected conspecifics, while genotype J8 has increased levels of male production in crowding conditions.

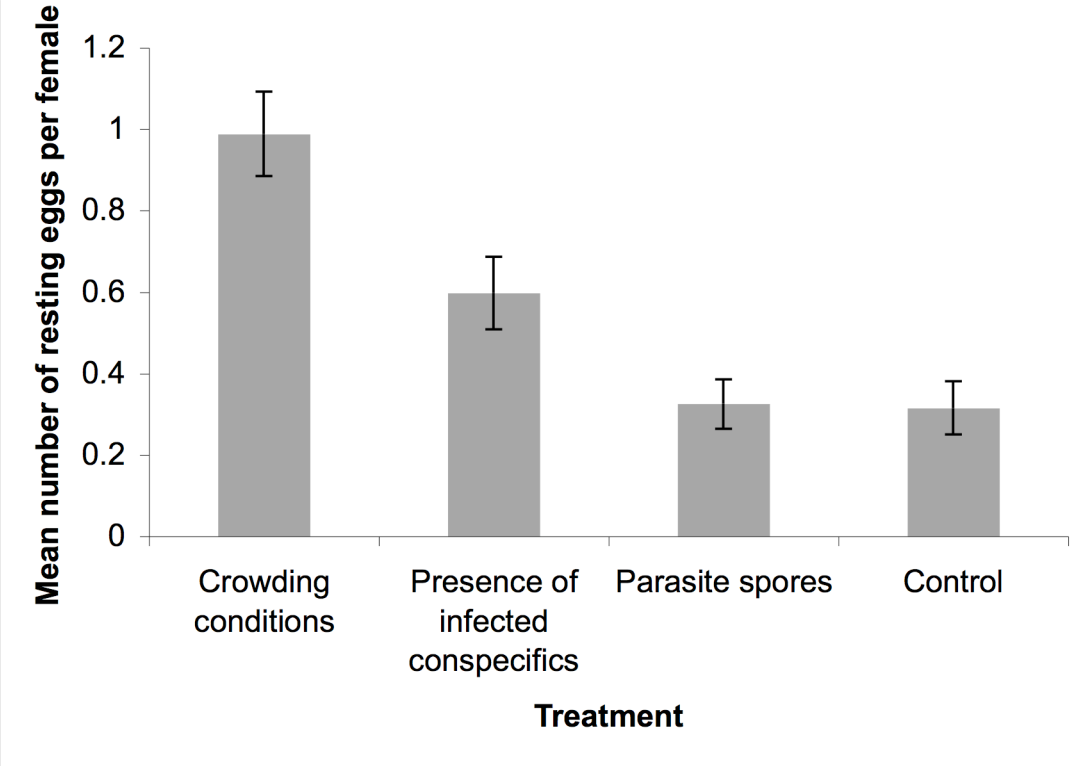

Figure 4.3. Mean levels of resting egg production per female for *Daphnia* exposed to conditions indicating the presence of infected conspecifics, crowding conditions, parasite spores, and normal water (± standard error). This figure shows the original untransformed data.

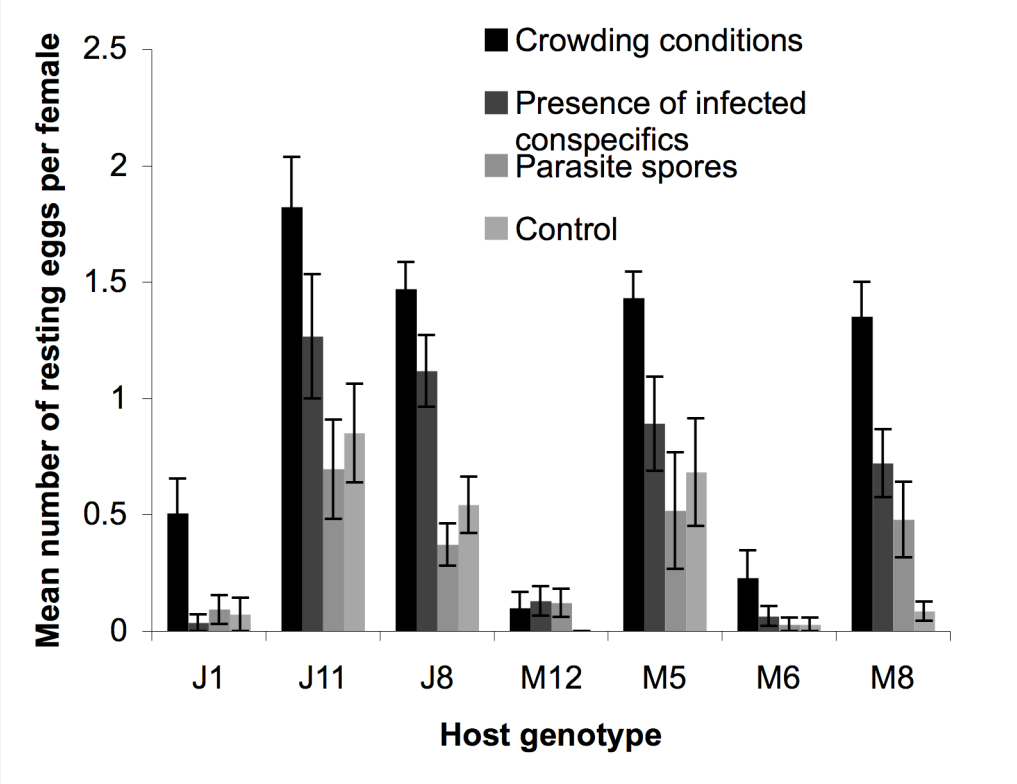

Figure 4.4. Mean levels of resting egg production by different *Daphnia* genotypes in different water treatments (± standard error). This figure shows original untransformed data.

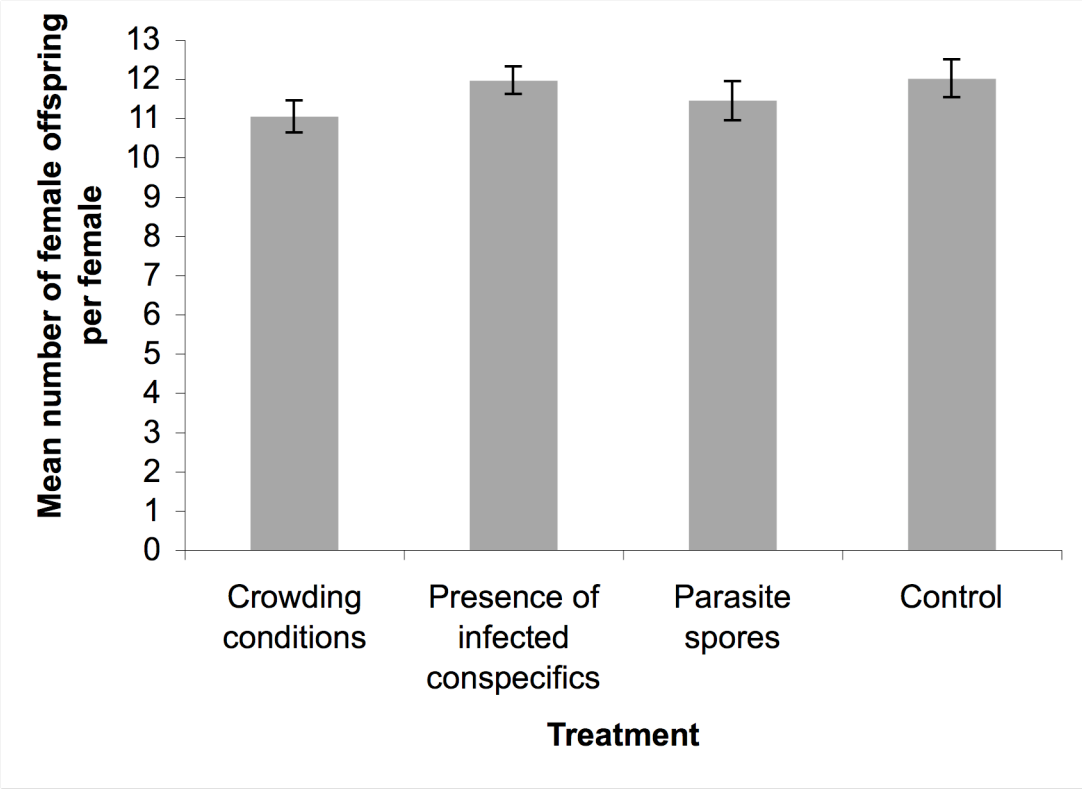

Figure 4.5. Mean levels of female production per female for *Daphnia* exposed to conditions indicating crowding conditions, the presence of infected conspecifics, parasite spores, and normal water (± standard error). This figure shows the original untransformed data.

4.5. Discussion

In this chapter I investigated the effects of 1) crowding conditions, 2) the presence of infected conspecifics and 3) direct exposure to parasite transmission spores on levels of sexual reproduction, (measured as levels of male and resting egg production), in the crustacean *Daphnia magna*. Both crowding conditions and the presence of infected conspecifics increased levels of male production, while increased levels of resting egg production was primarily due to crowding conditions. Regarding male production there was an interesting interaction between genotype and water type, showing that cues for the onset of male production are genotype specific. Regarding resting egg production, genotype specific effects were also detected, but these were less dramatic than that detected for male production (discussed below). I found no evidence to support enhanced numbers of males or resting eggs by parasite spores. In summary these results suggest that of the three factors I studied, crowding conditions are the strongest cue for resting egg production, whereas male production is additionally sensitive to whether cues indicate the presence of infected conspecifics, and that this is genotype dependent.

The genotype by environment interaction for male production shows that one genotype increases male production in response to crowding conditions, but not if the crowding conditions also indicate the presence of infected conspecifics. Two other genotypes have increased male production only if cues indicate the presence of infected conspecifics. Increased levels of resting egg production were largely due to some genotypes responding to crowding conditions to a greater degree than others. One genotype did though have enhanced levels of resting egg production in response to the presence of infected conspecifics. Thus, it is the interaction for male production that is particularly interesting because it reveals a crossing of reaction norms, and

emphasises the great extent to which responses in the field can be entirely genotype specific. Genotype by environment interactions for susceptibility have recently been shown to play a large, and possibly widespread role in determining the outcome of host-parasite interactions (Blanford *et al.*, 2003; Fels & Kaltz, 2006; Mitchell *et al.*, 2005a). Genotype by environment interactions for susceptibility coupled with 1) genotype by environment interactions for the onset of male and resting egg production, and 2) the genetic linkage between parasitism, sexual reproduction and resting egg production described in Chapter 2, creates an arena of great complexity for parasite mediated selection.

During the field studies (Chapters 2 and 3) male and resting egg production were observed in this population as parasite prevalence was increasing in the population, prior to the peak of the parasite epidemic, and when *Daphnia* population density was high. Peak levels of resting egg production coincided with peak population densities, which is consistent with the results of this experiment. Peak levels of male production were also observed when population densities were high, and before parasite prevalence could be accurately recorded, but when a low degree of pre-pathogenic infection was observed in the population. Cues for the onset of male production in this experiment are thus also consistent with conditions in the field. However both male and resting egg production will have been affected by other unmeasured variables in the field, so extrapolation from field to the laboratory requires caution.

It is particularly interesting that some genotypes can distinguish between cues indicating crowding conditions and crowding cues that also indicate the presence of infected conspecifics. Indeed, cues indicating the presence of infected conspecifics only differ to those indicating crowding conditions in that they are established using

infected, rather than uninfected, *Daphnia*. It would seem *Daphnia* are able to distinguish the presence of infected or healthy individuals through chemically mediated cues in the water. This is an exciting result, though one which has been observed in other species. For example, lobsters infected with a virus have been shown to induce behavioural changes in healthy conspecifics, which is thought to be chemically mediated (Behringer *et al.*, 2006).

These results may have important implications for parasite-mediated dynamics. The induction of sexual reproduction in response to increasing parasite prevalence is beneficial for more susceptible genotypes, because it provides a mechanism by which their offspring can escape an impending parasite epidemic. Studying the speed with which mates are found and resting eggs can be manufactured compared to how quickly an epidemic spreads would be key to determining the adaptive significance of using infected conspecifics as a cue to diapause. Generally though, if levels of sexual reproduction in some genotypes are induced by the presence of infected conspecifics in years when parasite presence is low, levels of sexual reproduction may also be low, which has population-wide consequences. Indeed Chapter 2 already confirmed that the genotypes (all of which tend to make resting eggs in the wild) used in this experiment were more susceptible to the parasite. It would be particularly interesting to explore this further and investigate the extent to which it is the most susceptible genotypes, compared to the most resistant, that respond to cues indicating the presence of infected conspecifics.

Chapter 5. General Discussion

There is an abundance of indirect evidence suggesting the widespread occurrence of parasite-mediated selection in natural populations, but few examples of parasite-mediated selection have been observed over time. This thesis therefore provides some of the best evidence for a response to parasite-mediated selection in a natural population. However, these dynamics may only have been detected due to the extreme temperatures experienced in 2003 (Chapters 2 and 3). This sort of nuance to host-parasite relationships may be key to why parasite-mediated responses to selection have not commonly been identified in host populations.

Despite the strong detrimental effects of parasites, the complexities associated with interactions between host and parasite genotypes, and their interaction with the environment, will almost certainly impact the likelihood of detecting parasite-mediated selection in the wild. This thesis revealed a further complexity that may interfere with the long-term study of host-parasite dynamics; that of a variety (Chapters 2 and 4) of genotype-specific links between sexual reproduction, which in *Daphnia* leads to the production of a resting egg, and parasite susceptibility. This result may explain why evidence for parasite-mediated selection in this system has tended to be weak, especially for studies that have investigated host-parasite dynamics across years, i.e. spanning a period of diapause (Little & Ebert, 2001). Specifically, if sampling of a population coincides with a recent emergence of genotypes from the resting egg bank, the genetic composition of the population is unlikely to reflect genetic change due to ongoing parasitism.

Future studies exploring parasite-mediated dynamics in the wild should simultaneously consider both the genetic components of the relationship, and how the

environment may influence dynamics. It has been relatively straightforward to recognise environmental factors that can affect responses to parasitism among different host genotypes in laboratory experiments. Future studies should, however, strive to relate observed host-parasite dynamics to environmental variation in the field. For instance, poor maternal environment can strongly enhance levels of offspring resistance in the *Daphnia*-parasite system (Mitchell & Read, 2005). Accordingly, future experimentation could investigate whether this response is subject to genotype by environment interactions, and whether parasite-mediated selection acts upon maternal effects. This could be done using the *Daphnia*-parasite system by stressing mothers collected before and after a natural parasite epidemic (using sampling strategies similar to those reported here), and comparing the resistance of their offspring to the parasite. Similar experiments involving *Daphnia* collected during high or low temperature periods in the field would be insightful.

Behavioural aspects of host-parasite dynamics, both parasite induced behavioural changes in the host (Biron *et al.*, 2005), and avoidance of sources of infection (Behringer *et al.*, 2006; Karvonen *et al.*, 2004) are likely to be mediated by environmental cues. It would be interesting to identify the extent to which behavioural differences in response to certain environmental conditions have a genetic basis, and explore how this behaviour may impact parasite-mediated dynamics in the wild. Experiments in the laboratory could identify the propensity for different genotypes to respond to certain environmental cues, and these findings applied to study in the field. There are many further aspects of behaviour or the environment that may affect the outcome of host-parasite interactions, but further studies on *Daphnia* would be well served by focusing on the interactions (those involving diapause, maternal effects, temperature and behaviour) the present thesis and other recent work has highlighted

as being likely to explain a substantial fraction of the variation we observe in this system. In this regard, an effort to formerly partition the variance observed in *Daphnia* infection experiments would also be welcomed.

The importance of interactions between particular host and parasite genotypes also requires further attention, in particular regarding how associations between host genotype by parasite genotype interactions may affect field dynamics. Laboratory experiments have, in a number of systems, established that the outcome of infection is determined by specific host genotype by parasite genotype interactions (Carius *et al.*, 2001; Salvaudon *et al.*, 2005). It would be interesting to investigate the extent to which particular host-parasite genotype interactions reflect dynamics predicted by the Red Queen Hypothesis. Such studies will require simultaneous temporal monitoring of both host and parasite genotypes, and it is conceivable that striking dynamics have been overlooked because temporal studies have typically lacked sufficient genetic resolution.

Temporal monitoring of clonal populations, like *Daphnia*, is made possible due to linkage disequilibrium whereby neutral molecular markers can be used to track whole genotype changes through time. However most organisms reproduce sexually which means gene complexes are broken up by recombination each generation, making it impossible to track associations between particular host and parasite genotypes (as measured by molecular markers). Studies aimed at investigating host-parasite co-evolution have therefore generally been limited to clonal organisms, but even these may have been limited by occasional bouts of sex. The identification of genes that are directly involved with host resistance and parasite infectivity will greatly facilitate the ability to explore parasite-mediated selection over time in both asexual and sexual organisms. The recognition of alleles that confer specific qualities

to hosts and parasites, as opposed to using neutral loci in linkage disequilibrium, will require increased genomic and post-genomic studies on organisms that are useful for the study of parasite-mediated dynamics in the field.

Clarification of the factors that influence parasite-mediated selection in the field generally will enable the successful implementation of disease management strategies and further understanding of factors that affect population dynamics. However, if we are to attain this, not only will we have to understand the consequences of host genotype by parasite genotype, and host genotype by environment interactions, but we may even have to unravel the daunting intricacies associated with three-way host genotype by parasite genotype by environment interactions. Further these factors may require assessment both within and across generations (Mitchell & Read, 2005). Future co-evolutionary studies should focus on confronting this complexity.

Appendix 1

A.1.1. Introduction

The following three experiments formed the groundwork for the main experiment described in Chapter 4. Here, I briefly describe the methods and results of each experiment, and give a short discussion summarising the findings. Sexual reproduction was observed in the field before the peak of the parasite epidemic, and also at a time when *Daphnia* population density was at its highest level (see results, Chapter 2). Although parasite prevalence could only be accurately recorded from the 27th June, some *Daphnia* infected with *Pasteuria ramosa* were observed in the population from the 13th June. The sole aim of the first experiment described below was to investigate whether parasite spores can enhance levels of sex. In this first experiment, cues that indicate crowding conditions are combined with other stimuli, in an attempt to induce sex. In contrast, the second two experiments explore whether crowding conditions or cues indicating the presence of infected conspecifics, may trigger sexual reproduction.

It has previously been found that the induction of sexual reproduction in *Daphnia* requires more than one stimulus. Accordingly, in each of these experiments, *Daphnia* are simultaneously exposed to a variety of stimuli known to be effective at inducing sexual reproduction. Intermediate intensities of the additional stimuli were chosen to allow room for the parasite related cues to further modify levels of sex.

A.1.2. Methods

A.1.2.1. Investigation of whether parasite presence enhances sexual reproduction in Daphnia?

Crowding conditions (Kleiven *et al.*, 1992) and the threat of predation (Slarsarczyk *et al.*, 2005) are two cues that have previously been shown to strongly induce sexual

reproduction in *Daphnia*. I therefore combined cues indicating crowding and the threat of predation in to a water treatment, termed crowded-fish water, and compared levels of sexual reproduction with *Daphnia* in normal water, with and without parasites.

The threat of predation was simulated by preparing water containing fish kairomones. Twenty-five three spined sticklebacks (*Gasterous aculeatus*) were kept in a 50 litre tank and fed de-frosted blood worms *ad libitum*. Half the water was collected daily from the tank, and replaced with clean water. The collected water was filtered through a 0.2 μm Wattman filter to remove debris. Methods of preparation for crowded water were similar to those described in the main experiment in Chapter 4. Cues indicating the threat of predation and crowding were combined by mixing *Daphnia* crowded water, and water containing fish kairomones at a 50:50 ratio daily, throughout the duration of the experiment. All treatments were subject to a fixed photoperiod of 11:13 hour L:D cycle, kept at a constant temperature of 20 C°, and following the infection period, each *Daphnia* fed 2 x 10^6 cells per day. These conditions were chosen to be optimal for intermediate levels of sexual reproduction (Kleiven *et al.*, 1992; Stross & Hill, 1965).

Three replicates of 21 clones were used in this experiment. From each replicate, groups of 5 offspring, always from the same clutch, were randomly assigned to each of 4 treatments; 1) Normal water with parasites, 2) Normal water with no parasites, 3) Crowded-fish water with parasites and 4) Crowded-fish water with no parasites. The experiment was set up over 5 days. Although only the 'with parasite' treatments received parasites, all treatments were subject to the same infection routine described in detail in Chapter 2. Briefly, each replicate was placed in a 60ml jar with sand at the bottom, containing the appropriate water type, and 1 x 10^5 parasite spores

were added to treatments 1) and 3). All jars were stirred daily for 8 days, and all subjected to a low feeding regime. On day 8 each group of 5 females was moved to a 200 mL jar containing either normal water, or crowded-fish water, depending on the treatment. Every other day, from day 8 onwards, each group of 5 females was moved to a clean jar containing the appropriate type of water. At each water change, when offspring were present in a jar they were removed, sexed and counted. The presence of resting eggs was also recorded. The experiment finished on day 34, at which time the proportion of *Daphnia* infected was recorded for the treatments that received parasites.

A.1.2.2. Can the presence of infected conspecifics trigger sexual reproduction; part I?

To create an environment that had previously contained infected individuals I collected water from all replicates in Treatment 1 (Normal water, with parasites), Experiment 1.2.1., from Day 8 to the end of the experiment. This created an environment of approximately 25 *Daphnia* per litre that had been exposed to the parasite and a substantial proportion of which were infected (mean infection level in treatment 1, Experiment 1, was 0.66 (± 0.06 SE)). In the same way, as a control, I collected water from all replicates in Treatment 2, thus creating crowding conditions of the same density using healthy individuals. Water from Treatments 1 and 2, from Experiment 1, were filtered using a 0.2 μm Wattman filter, and stored separately in the dark until needed.

Eighteen clones were used in this experiment. From each replicate of each clone, groups of 5 offspring, always from the same clutch, were randomly assigned to 3 water treatment types; 1) presence of infected conspecifics, 2) crowding conditions

and 3) normal water. This experiment was set up over 4 days. Methods were identical to those described in Experiment A.1.2.1, except that none of the treatments received parasites. This experiment finished on day 22.

A1.2.3. Can the presence of infected conspecifics trigger sexual reproduction; part II?

Experiment A1.2.2. was repeated, but in order to overcome the problem of no or low levels of sexual reproduction (see results, Experiment A1.2.1. and A1.1.2.2.) I altered experimental conditions. It is possible that it is not stressful (low food) conditions that induce sex, but rather, changing conditions, so in this experiment I use a new food regime, which I term variable.

From each replicate of each clone, groups of 5 offspring, always from the same clutch, were randomly assigned to 3 water treatment types; 1) presence of infected conspecifics, 2) presence of infected conspecifics and 3) normal water. Methods of preparation for water containing cues indicating the presence of infected conspecifics and crowded conditions were identical to those described in Experiment A1.2.2. On day 1 all treatments were placed in a 200mL jar, and each *Daphnia* fed 3.5 x 10^6 algal cells per day until the first clutch of offspring was observed for each replicate. After this point the food level was reduced to 1.5 x 10^6 algal cells per *Daphnia* per day. Water was changed on day 5, and three times a week following observation of the first clutch. Replicates were maintained in a 20 C° environment and subjected to a 16:8 hour L:D cycle. All offspring were counted and sexed, and the presence of resting eggs recorded. The experiment finished on day 30.

A1.2.4. Analysis

I analysed the presence/absence of sexual reproduction using contingency table analysis in Experiments A1.2.1. and A1.2.3. (no sexual reproduction was recorded in Experiment A1.2.2.). The presence of sexual reproduction was determined by recording whether each clone, in each treatment group, produced males and/or resting eggs.

I used analysis of variance to investigate whether mean total offspring production per female was affected by water type and infection, and to establish whether the proportion of *Daphnia* infected was affected by water type in Experiment A1.2.1. Offspring data was square root transformed, and infection data arcsine-square root transformed to meet the assumptions of ANOVA. Analysis was performed on means obtained for each clone, for each of the three replicates, in each treatment group. I used a repeated measures ANOVA to investigate whether there was a difference in early reproduction and late reproduction between the different treatment groups. Early reproduction was defined as the mean number of offspring produced between day 14 (when the first clutches of offspring were observed) and day 22, and late reproduction as the mean number of offspring between day 24 and day 32. Reproduction on day 34 was excluded from this analysis so that early and late reproduction could be compared over equal periods of time.

I used analysis of variance to investigate whether water type affected mean offspring production per female in Experiments A1.2.2. and A1.2.3. Untransformed data was used for both these analyses since the data met the assumptions of ANOVA. 'Set up day' was included in the model as a random effect for analysis of Experiment A1.2.2., but could not be included for Experiment A1.2.3 as replicates did not contribute to each treatment group, on each day. I also used analysis of variance to

investigate whether water type affected offspring sex ratio in Experiment A1.2.3. Sex ratio data was arcsine transformed to meet the assumptions of ANOVA. All analysis was carried out in JMP 5.1.

A1.3. Results

In Experiment A1.2.1. neither parasite presence nor crowded-fish water affected levels of sexual reproduction alone, or in combination (Fig A1.1; $\chi^2 = 2.63$, df = 3, p = 0.45). Females kept in crowded-fish water had higher mean total offspring production than females kept in normal water (Fig A1.2; $F_{1, 80} = 14.11$, p = 0.0003). Reflecting a cost of infection, females that received the parasite had lower mean total offspring production than females that did not receive the parasite (Fig A1.2; $F_{1, 80} = 130.33$, $p < 0.0001$). The interaction between water treatment type and parasite presence was not significant ($F_{1, 80} = 0.80$, p = 0.37) and water treatment type did not affect the mean proportion of *Daphnia* that became infected ($F_{1, 40} = 0.03$, p = 0.85). *Daphnia* in CF-water had higher levels of early reproduction than *Daphnia* in normal water (Fig A1.3; $F_{1, 80} = 6.09$, p = 0.02). *Daphnia* in the presence of the parasite had higher levels of early reproduction than late reproduction (Fig A1.3; $F_{1, 80} = 17.42$, $p < 0.0001$). The interaction between late versus early reproduction, water treatment type and infection was not significant ($F_{1, 80} = 0.61$, p 0.44).

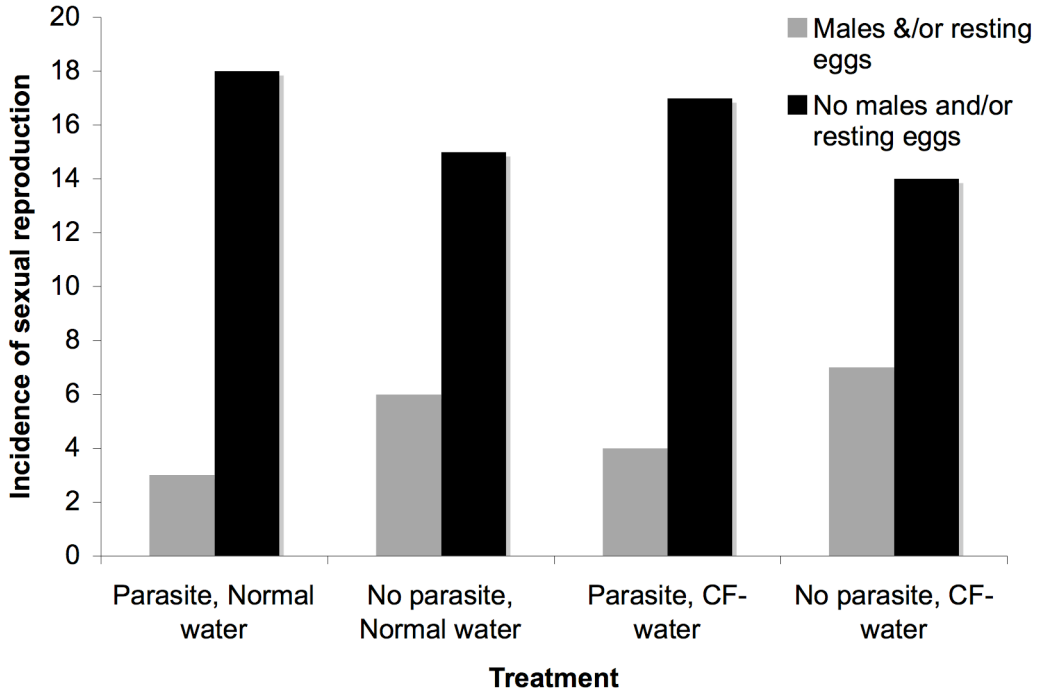

Figure A1.1. Incidence of sexual reproduction (measured as occurrence of males and/or resting eggs) among the different clones in normal and crowded-fish water (CF-water), with and without the parasite.

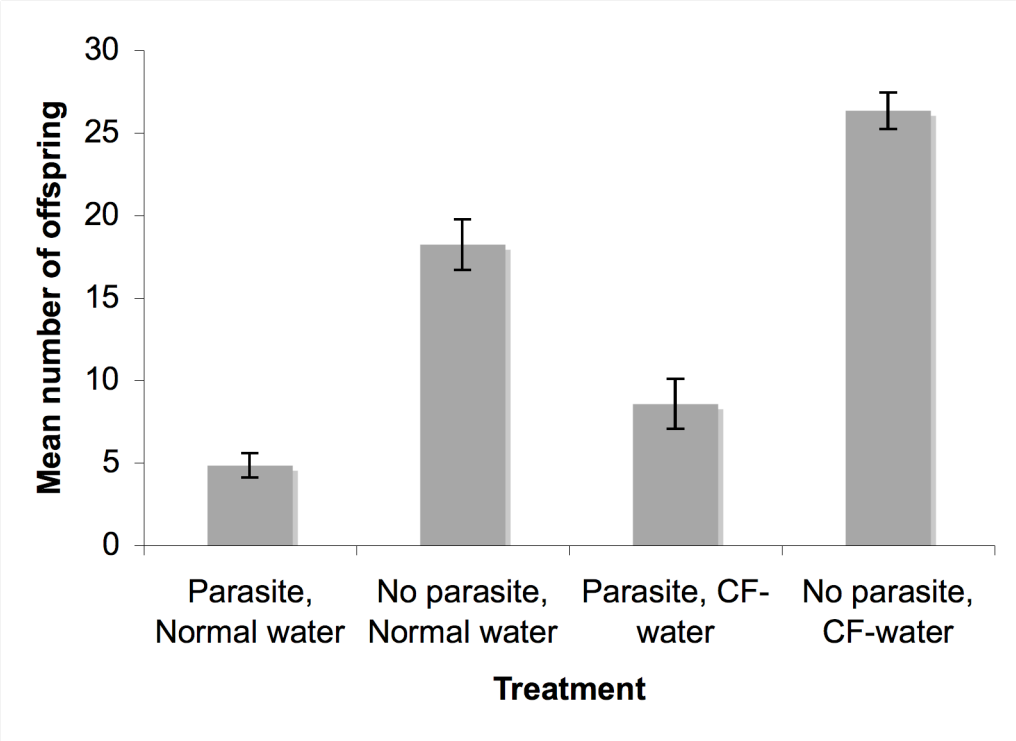

Figure A1.2. Mean total offspring production per female in normal and CF-water, with and without the parasite (± standard error). This figure shows original untransformed data.

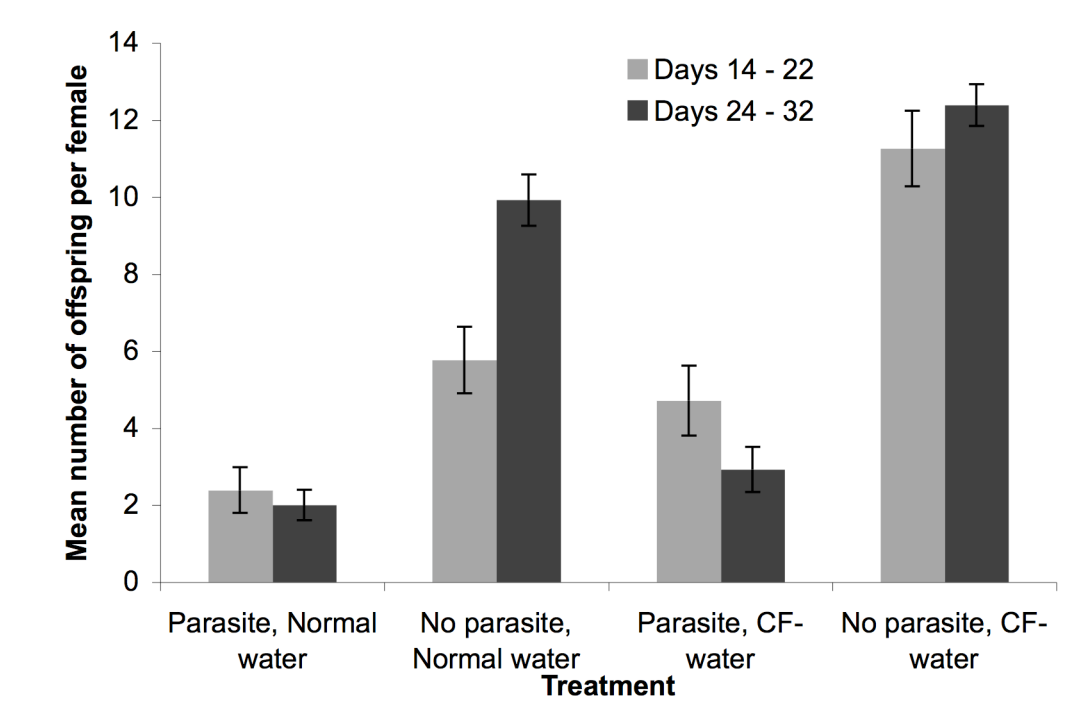

Figure A1.3. Comparison of early versus late reproduction for females in the different water treatment types (± standard error). This figure shows original untransformed data.

The incidence of sexual reproduction did not differ across the different water treatments in Experiment A1.2.3. (Fig A1.4; $\chi^2 = 5.21$, df = 2, p = 0.07). No sexual reproduction was observed in Experiment A1.2.2. There was no difference in offspring production between females in water indicating the presence of infected conspecifics, crowding conditions, or normal water in Experiment A.1.2.2. ($F_{2, 47} = 2.66$, p = 0.08), or Experiment A1.2.3. ($F_{2, 36} = 0.93$, p = 0.40), and water type did not affect offspring sex ratio ($F_{2, 36} = 1.08$, p = 0.35) in Experiment A1.2.3.

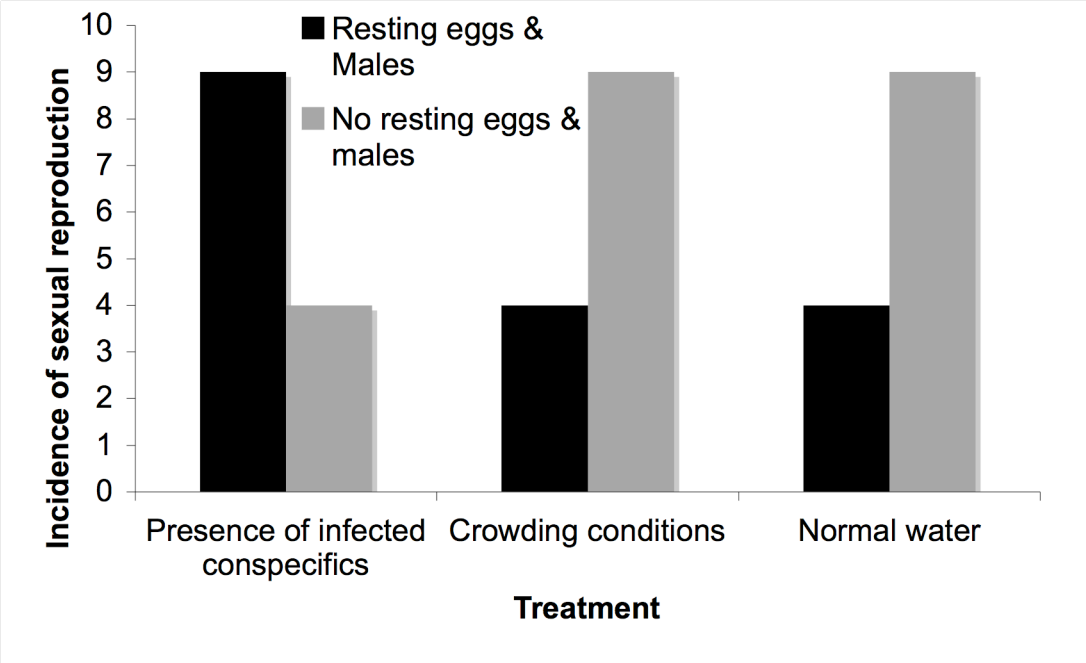

Figure A1.4. Incidence of sexual reproduction for females in the presence of infected conspecifics, crowing conditions and normal water in Experiment 3.

A1.4. Summary of findings from initial experimentation

These three experiments explored whether parasite spores, the presence of infected conspecifics, or crowding conditons, could act as a stimulus for sexual reproduction alone, or through interaction with other environmental stimuli. Sexual reproduction and the production of resting eggs was not enhanced by any of the parasite treatments, or by crowding conditions. The only discernable trend was for higher levels of sexual reproduction for individuals simultaneously exposed to water that had contained infected individuals and a variable food regime in Experiment A1.2.3. (Fig A1.4.), but this result was not significant. In Experiment A1.2.1. the incidence of sexual reproduction was low across all treatments (Fig A1.1.), whereas no males or resting eggs were observed at all in Experiment A1.2.2.

Induction of sexual reproduction in *Daphnia* typically requires a variety of stimuli (Kleiven *et al.*, 1992; Slarsarczyk *et al.*, 2005; Stross & Hill, 1965). It may be that the stimuli used in these experiments were inappropriate, or were at the wrong levels for this population. The fact that levels of sexual reproduction were low across all experiments suggests that even if parasites do enhance sexual reproduction, the power to explore their effects in these experiments was limited.

Aside from sexual reproduction, the trend for higher levels of early reproduction by *Daphnia* in crowded-fish water does raise some interesting questions. Although this response cannot be attributed to either the presence of fish kairomones, or chemicals produced by high numbers of *Daphnia*, higher levels of early offspring production are consistent with the presence of a predation threat (Sakwinska, 1998, 2002). Moreover, I found no evidence in Experiments A1.2.2. and A1.2.3. that the presence of conspecifics alone resulted changes in offspring production. Early reproduction is thought to be beneficial in the presence of predators, in part to ensure

reproductive success (Sakwinska, 1998, 2002), but also because smaller adult size, which is associated with earlier maturation and reproduction, reduces the chance of detection by predators (Brooks & Dodson, 1965). In contrast to this, however, crowding conditions have been found to result in the production of fewer, larger offspring in *Daphnia* (Burns, 1995; Cleuvers *et al.*, 1997). It has been suggested that crowding conditions may be a signal of imminent competition for food. Crowding conditions, akin to low food conditions, are therefore thought to trigger the production of higher quality offspring capable of surviving periods of starvation (Cleuvers *et al.*, 1997), although evidence regarding the survival ability of offspring produced under such conditions is inconsistent (Cleuvers *et al.*, 1997; Guinnee *et al.*, Submitted). If *Daphnia* are able to detect both cues in the water, then these results indicate that the response to predation is much stronger than the response to crowding conditions.

Appendix 2. Swimming Pools, Suicide and Sex Parties: How a Hairworm Finds a Mate

The following article received a runners-up prize for The Daily Telegraph and Bayer Science Writer Awards 2006.

Growing up is hard to do whether it's due to acne, leaving your mother's pouch or metamorphosing in to a butterfly. Hairworms however have a remarkable way of dealing with the problems usually associated with growing up. After beginning life as free-living aquatic juveniles, hairworms become parasitic, sharing their adolescent growing pains with an insect or spider host. They reside within their host, where they enjoy a constant supply of food. During this parasitic phase hairworms can grow up to three or four times the length of their host. However, once the worms reach adulthood they must return to water to find a mate. To achieve this they manipulate the behaviour of their host by releasing chemicals that persuade them to jump in to water and take a fatal swim. Once in the water, the hairworm will then pierce a tiny hole in the host's body, from which it will eventually emerge and swim off to find a mass of mating hairworms. Unfortunately the hapless host does not usually survive this ordeal and drowns.

Frederic Thomas and colleagues at the Institute of Research and Development in Montpellier set out to investigate this phenomenon following anecdotal observations of this bizarre behaviour in the wild. They observed a total of nine species of hosts exhibiting this behaviour, including three types of spider and two types of cricket.

To establish whether the hairworm directly causes the suicidal behaviour of the host they set up a cordon around a swimming pool at one of the team's houses and intercepted crickets venturing in to the area. At the same time they also collected crickets found in a forest near to the pool. The following day they simultaneously released pairs of crickets found in the forest and pairs found near to the swimming pool. They placed them two metres from the edge of the pool and waited. The behavioural difference between infected and uninfected crickets was quite remarkable. The research team found that 48% of crickets harbouring a worm jumped in to the pool within 15 minutes, compared to only 13% of uninfected crickets and that 95% of crickets collected near to the pool were infected with a hairworm, while only 15% of crickets found in the forest were infected. These differences are quite astonishing and strongly suggest that infected crickets are attracted to the water in the swimming pool.

However, Thomas and his co-workers needed more evidence to support this idea. They collected more crickets from beside the pool and the forest and took them back to the laboratory. Each cricket was placed at the entrance to a maze with two arms, one leading to a trough containing water, the other leading to an empty trough. The arm each cricket chose was completely random which did not seem to support the idea that infected crickets are attracted to water. There was, however, a behavioural difference between infected and uninfected crickets when they encountered water. All infected crickets that encountered the trough containing water jumped in, compared to only one uninfected cricket out of twelve. So how does an adolescent hairworm persuade their host to commit suicide?

They investigated this using proteomics, an approach that allows the identification of protein molecules in both the cricket and the hairworm. Intriguingly they found that both infected crickets, and their parasitic hairworms expressed different protein molecules during the manipulative phase. Most interesting was the finding that hairworms, during this period, produce protein molecules that mimic those of the cricket. These mimetic proteins belong to a family that play an important role in the development of the cricket's central nervous system. This provides compelling evidence that the hairworm actively produces chemicals that directly alters cricket behaviour, causing them to perform this suicidal behaviour.

The final piece in the puzzle comes once the cricket is in the water as it can take up to 10 minutes for the hairworm to emerge. This can be a risky business, as the cricket and the emerging worm will be very conspicuous to predators. Dr. Thomas investigated this in the laboratory and unsurprisingly found that crickets infected with, or expelling hairworms were easy prey for their predators, such as frogs or fish. Amazingly though, the hairworm often managed to escape predation by wriggling from the mouth, nose or gills of the predator that had consumed their cricket host. However, if the hairworm did not appear within five minutes of predation it met the same fate as its host, failing to meet its destiny of swimming off to find a mate.

(767 words)

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